FORM PTO-1390 (REV. 5/93)	U.S. Department of Commerce Patent and Trademark Office	Attorney's Docket Number 960-29
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. Application No. (If known, see 37 C.F.R. 1.5) 08/886734

International Application No.	International Filing Date	Priority Date Claimed
PCT/EP95/04575	21 November 1995	22 November 1994

Title of Invention

LGMD GENE CODING FOR A CALCIUM DEPENDENT PROTEASE

Applicant(s) For DO/EO/US

BECKMANN et al

Applicant herewith submits to the United States Designed/Elected Office (DO/EO/US) the following items and other information:

1. [x] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [x] This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [x] A proper Demand for International preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [x] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371 (c)(2)).
7. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau
   c. [ ] have not been made; however, the time limit for making such amendments has **NOT** expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).
11. The above checked items are being transmitted:
    a. [ ] before the 18th month publication.
    b. [ ] after publication and the Article 20 communication but before 20 months from the priority date.
    c. [ ] after 20 months.
    d. [x] by 30 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
    e. [ ] after 30 months.

    Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted (1) after 20 months and no proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date, or (2) after 30 months and a proper demand for International preliminary Examination was made by 19 months from the earliest claimed priority date.
12. At the time of transmittal, the time limit for amending claims under Article 19
    a. [ ] has expired and no amendments were made.
    b. [ ] has not yet expired.
13. [ ] Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on _____, namely:

Items 14. to 19. below concern other document(s) or information included:

14. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
15. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
16. [x] A **FIRST** preliminary amendment.
    [ ] A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. [ ] A substitute specification.
18. [ ] A change of power of attorney and/or address letter.

Attorney's Docket Number **960-29**

19. [**x**] Other items or information:

International Search Report

20. [**X**] The following fees are submitted:	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): -- Search Report has been prepared by the EPO or JPO ..... $910.00 -- International preliminary examination fee paid to USPTO (37 CFR 1.492)..... $700.00 -- No international preliminary examination fee paid to USPTO (37 CFR 1.492) but international search fee paid to USPTO (37 CFR 1.445 (a)(2))..... $770.00 -- Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... $1,040.00 -- International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provision of PCT Article 33(1) to (4) $96.00		

ENTER APPROPRIATE BASIC FEE AMOUNT =						$	**910.00**	
Surcharge of $130.00 for furnishing the National fee or oath or declaration later than [ ] 20 [**x**] 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).						$	**130.00**	
CLAIMS	NUMBER FILED		NUMBER EXTRA		RATE			
Total Claims	**22**	- 20 =	**2**	X	$ 22.00	$	**44.00**	
Independent Claims	**4**	- 3 =	**1**	X	$ 80.00	$	**80.00**	
Multiple Dependent Claim(s) (if applicable)					+ $260.00	$	**260.00**	
TOTAL OF ABOVE CALCULATIONS =						$	**1,424.00**	
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28).						$		
SUBTOTAL =						$	**1,424.00**	
Processing fee of $130.00, for furnishing the English Translation later than [ ] 20 [ ] 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).						$		
TOTAL NATIONAL FEE =						$	**1,424.00**	
Fee for recording the enclosed assignment (37 CFR1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +						$		
Fee for Petition to Revive Unintentionally Abandoned Application ($1,290 -- Small Entity Fee = $645)						$		
TOTAL FEES ENCLOSED =						$	**1,424.00**	
						Amount to be refunded	$	
						Charged	$	

a. [**X**] A check in the amount of.....$ 1,424.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. 14-1140 in the amount of $ _____ to cover the above fees. A duplicate copy of this form is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140 . A duplicate copy of this form is enclosed.

SEND ALL CORRESPONDENCE TO:

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32,955 May 22, 1997
Registration Number Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

BECKMANN et al Atty. Ref.: **960-29**

Serial No. **(To Be Assigned)** Group:

Filed: **22 May 1997** Examiner:

For: **LGMD GENE CODING FOR A CALCIUM DEPENDENT PROTEASE**

* *

May 22, 1997

Honorable Commissioner of Patents
and Trademarks
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

In order to place the above-identified application in better condition for examination, please amend the above-identified application as follows:

IN THE CLAIMS:

Claim 3, line 2, delete "or 2".

Claim 5, line 2, change "claims 1 to 4" to -- Claim 1 --.

Claim 6, line 1, delete "or 6".

Claim 7, line 1, delete "or 6".

Claim 8, line 3, change "any one of claims 1 to 4" to -- Claim 1 --.

Claim 10, line 1, change "claims 5 to 6" to -- Claim 5 --.

Claim 11, line1, change "claims 10 or 11" to -- Claim 10 --.

Claim 12, line 1, change "claims 5 to 7" to -- Claim 5 --.

Claim 13, line 1, change "one of claims 1 to 4" to -- Claim 1 --.

Claim 17, line 1, delete "or 16".

Claim 20 (Amended) Pharmaceutical composition for the treatment of an LGMD2 disease characterized in that [in] it contains a component selected from the group of:

a) a nucleic acid sequence according to claim[s] 1 [to 4],

b) a host cell according to claim 8,

c) an aminoacid sequence according to claim[s] 5 [to 7].

REMARKS

The above amendments are made to place the claims in a more traditional format.

Respectfully submitted,

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LGMD gene coding for a calcium dependent protease

The invention relates to the isolated gene coding for a calcium dependent protease belonging to the Calpaïn family which, when it is mutated, is a cause of a disease called Limb-Girdle Muscular Dystrophy (LGMD).

The term limb-girdle muscular dystrophy (LGMD) was first proposed by Walton and Nattrass (1954) as part of a classification of muscular dystrophies. LGMD is characterised by progressive symmetrical atrophy and weakness of the proximal limb muscles and by elevated serum creatine kinase. Muscle biopsies demonstrate dystrophic lesions and electromyograms show myopathic features. The symptoms usually begin during the first two decades of life and the disease gradually worsens, often resulting in loss of walking ability 10 or 20 years after onset (Bushby, 1994). Yet, the precise nosological definition of LGMD still remains unclear. Consequently, various neuromuscular diseases such as facioscapulohumeral, Becker muscular dystrophies and especially spinal muscular atrophies have been occasionally classified under this diagnosis. For example, a recent study (Arikawa et al., 1991) reported that 17% (out of 41) of LGMD patients showed a dystrophinopathy. These issues highlight the difficulty in undertaking an analysis of the molecular and genetic defect(s) involved in this pathology.

Attempts to identify the genetic basis of this disease go back over 35 years. Morton and Chung (1959) estimated that "the frequency of heterozygous carrier ... is 16 per thousand persons". The same authors also stated that "the segregation analysis gives no evidence on whether these genes in different families are allelic or at different loci". Both autosomal dominant and recessive transmission have been reported, the latter being more common with an estimated prevalence of 10^{-5} (Emery, 1991). The localisation of a gene for a recessive form on chromosome 15 (LGMD2A, MIM 253600; Beckmann et al., 1991) provided the definitive proof that LGMD is a specific genetic entity. Subsequent genetic analyses confirmed this chromosome 15 localisation (Young et al., 1992; Passos-Bueno et al., 1993), the latter group demonstrating genetic heterogeneity of this disease. Although a recent study localised a second mutant

gene to chromosome 2 (LGMD2B, MIM 253601; Bashir et al., 1994), there is evidence that at least one other locus can be involved.

Genetic analyses of the LGMD2 kindreds revealed unexpected findings. First genetic heterogeneity was demonstrated in the highly inbred Indiana Amish community. Second although the Isle of la Réunion families were thought to represent a genetic isolate, at least 6 different disease haplotypes were observed , providing evidence against the hypothesis of a single founder effect (Beckmann et al., 1991) in this inbred population.

The nonspecific nosological definition, the relatively low prevalence and genetic heterogeneity of this disorder limit the number of families which can be used to restrict the genetic boundaries of the LGMD2A interval. Cytogenetic abnormalities, which could have helped to focus on a particular region, have not been reported. Immunogenetic studies of dystrophin-associated proteins (Matsumura et al., 1993) and cytoskeletal or extracellular matrix proteins such as a merosin (Tomé et al., 1994) failed to demonstrate any deficiency. In addition, there is no known specific physiological feature or animal model that could help to identify a candidate gene. Thus, there is no alternative to a positional cloning strategy.

It is established that the LGMD2 chromosomal region is localized on chromosome 15 as 15q15.1 - 15q21.1 region (Fougerousse et al., 1994).

Construction and analysis of a 10-12 Mb YAC contig (Fougerousse et al., 1994) permitted the mapping of 33 polymorphic markers within this interval and to further narrow the LGMD2A region to between D15S514 and D15S222. Furthermore, extensive analysis of linkage disequilibrium suggested a likely position for the gene in the proximal part of the contig.

The invention results from the construction of a partial cosmid map and the screening by cDNA selection (Lovett et al., 1991 ; Tagle et al., 1993) for muscle-expressed sequences encoded by this interval led to the identification of a number of potential candidate genes. One of these, previously cloned by Sorimachi et al. (1989), encodes a muscle specific protein, nCL1 (novel Calpain Large subunit 1), which belongs to the calpain family (CANP, calcium-activated neutral protease; EC 3.4.22.17), and appeared to be a functional candidate gene for this disease.

Calpains are non-lysosomal intracellular cysteine proteases which require calcium for their catalytic activities (for a review see Croall D.E. et al. 1991). The mammalian calpains include two ubiquitous proteins CANP1 and CANP2 as well as tissue-specific proteins. In addition to the muscle specific nCL1, stomach specific nCL2 and nCL2' proteins have also been described; these are derived from the same gene by alternative splicing. The ubiquitous enzymes consist of heterodimers with distinct large subunits associated with an common small subunit ; the association of tissue-specific large subunits with a small subunit has not yet been demonstrated. The large subunits of calpains can be subdivided into 4 protein domains. Domains I and III, whose functions remain unknown, show no homology with known proteins. Domain I, however, seems important for the regulation of the proteolytic activity. Domain II shows similarity with other cysteine proteases, sharing histidine, cysteine and asparagine residues at its active sites. Domain IV comprises four EF-hand structures which are potential calcium binding sites. In addition, three unique regions with no known homology are present in the muscle-specific nCL1 protein, namely NS, IS1 and IS2, the latter containing a nuclear translocation signal. These regions may be important for the muscle specific function of nCL1.

It is usually accepted that muscular dystrophies are associated with excess or deregulated calpains, and all the known approaches for curing these diseases are the use of antagonists of these proteases ; examples are disclosed in EP 359309 or EP 525420.

The invention results from the finding that, on the opposite to all these hypothesis, the LGMD2 disease is strongly correlated to the defect of a calpain which is expressed in healthy people.

The invention relates to the nucleic acid sequence such as represented in Figure 2 coding for a Ca^{++} dependent protease, or calpain, which is involved in LGMD2 disease, and more precisely LGMD2A. It also relates to a part of this sequence provided it is able to code for a protein having a calcium-dependent protease activity involved in LGMD2, or a sequence derived from one of the above sequences by substitution, deletion or addition of one or more nucleotides provided that said sequence is still coding for said protein, all the nucleic acids yielding a sequence complementary to a sequence as defined above.

The genomic organisation of the human nCL1 gene has been determined by the inventors, and consists of 24 exons and extends over 40 kb as represented in Figure 8, and is also a part of the invention. About 35 kb of this gene have been sequenced. A systematic screening of this gene in LGMD2A families led to the identification of 14 different mutations, establishing that a number of independent mutational events in nCL1 are responsible for LGMD2A. Furthermore, this is the first demonstration of a muscular dystrophy resulting from an enzymatic rather than a structural defect.

In the present specification, CANP3 means the protein which is a Ca^{++} dependent protease, or calpaïn, and coded by the nCL1 gene on chromosome 15.

The invention relates also to a protein, called CANP3, consisting in the amino acid sequence such as represented in figure 2 and which is involved, when mutated, in the LGMD2 disease.

The cDNA of the gene coding for CANP3, which is coding for the protein, is also represented in Figure 2, and is a part of the invention.

The protein coded by this DNA is CANP3, a calcium-dependent protease belonging to the Calpaïn family.

Are also included in the present invention the nucleic acid sequences derived from the cDNA of Figure 2 by one or more substitutions, deletions, insertions, or by mutations in 5' or 3' non coding regions or in splice sites, provided that the translated protein has the protease, calcium-dependent activity, and when mutated, induce LGMD2 disease.

The nucleic acid sequence encoding the protein might be DNA or RNA and be complementary to the nucleic and sequence represented in Figure 2.

The invention also relates to a recombinant vector including a DNA sequence of the invention, under the control of a promoter allowing the expression of the calpaïn in an appropriate host cell.

A procaryotic or eucaryotic host cell transformed by or transfected with a DNA sequence comprising all or part of the sequence of Figure 2 is a part of the invention.

Such a host cell might be either :

- a cell which is able to secrete the protein and. this recombinant protein might be used as a drug to treat the LGMD2, or

- a packaging cell line transfected by a viral or retroviral vector : the cell lines bearing recombinant vector might be used as a drug for gene therapy of LGMD2.

All the systems used today for gene therapy including adenoviruses and retroviruses and others described for example in « l'ADN médicament », (John Libbey, Eurotext, 1993), and bearing one of the DNA sequence of the invention are included herein by reference.

The examples hereunder and attached figures indicate how the structure of the gene was established, and how relationship between the gene and the LGMD was established.

Legend of the figures :

Figure 1:

A) Genomic organisation of the nCL1 gene

The gene covers a 40 kb region of which 35 were sequenced (Accession number pending). Introns and exons are drawn to scale, the latter being indicated by numbered vertical bars. The first intron is the largest one and remains to be fully sequenced. Position of intragenic microsatellites are indicated by asterisks. Arrows indicate the orientation of Alu (closed) and of Mer2 (greyed) repeat sequences.

B) *EcoR*I restriction map

An *EcoR*I (E) restriction map of this region was established with the help of cosmids from this region. The location of nCL1 gene is indicated as a black bar. The size of the corresponding fragments are indicated and are underlined when determined by sequence analysis.

C) Cosmid map of the nCL1 gene region.

Cosmids were from a cosmid library constructed by subcloning YAC 774G4 (Richard in preparation) and are presented as lines. Dots on lines indicate positive STSs (indicated in boxed rectangles). A minimum of three cosmids cover the entire gene. T3,T7

Figure 2: Sequence of the human nCL1 cDNA (B) , and the flanking 5' (A) and 3' (C) genomic regions

A) and C) The polyadenylation signal and putative CAAT, TATAA sites are boxed. Putative Sp1 (position -477 to -472), MEF2 binding sites (-364 to -343) and CArG box (-685 to -672) are in bold. The Alu sequence present in the 5' region is underlined

B) The corresponding amino acids are shown below the sequence. The coding sequence between the ATG initiation codon and the TGA stop codon is 2466 bp, encoding for a 821 amino acid protein. The adenine in the first methionine codon has been assigned position 1. Locations of introns within the nCL1 gene are indicated by arrowheads. Nucleotides which differ from the previously published ones are indicated by asterisks.

Figure 3: Alignments of amino acid sequences of the muscle-specific calpains.

The human nCL1 protein is shown on the first line. The 3 muscle-specific sequences (NS, IS1 and IS2) are underlined. The second line corresponds to the rat sequence (Accession no P). The third and fourth lines show the deduced amino acid sequences encoded by pig and bovine Expressed Sequences Tagged (GenBank accession no U05678 and no U07858, respectively). The amino acids residues which are conserved among all known members of the calpains are in reverse letters. A period indicates that the same amino acid is present in the sequence. Letters refer to the variant amino acid found in the homologous sequence. Position of missense mutations are given as numbers above the mutated amino acid.

Figure 4: Distribution of the mutations along nCL1 protein structure.

A) Positions of the 23 introns are indicated by vertical bars in relation to the corresponding amino acid coordinates.

B) The nCL1 protein is depicted showing the four domains (I, II, III, IV) and the muscle specific sequences (NS, IS1 and IS2). The position of missense mutations within nCL1 domain are indicated by black dots. The effect of nonsense and frameshift mutations are illustrated as truncated lines, representing the extent of protein synthesised. Name of the corresponding families are indicated on the left of the line. The out of frame ORF is given by hatched lines.

Figure 5: Northern blot hybridisation of a nCL1 clone

A mRNA blot (Clontech) containing 2 µg of poly(A)+ RNA from each of eight human tissues was hybridised with a nCL1 genomic clone spanning exons 20 and 21. The latter detects a 3.6 kb mRNA present only in a line corresponding to the skeletal muscle mRNA.

Figure 6: Representative mutations identified by heteroduplex analysis.

Examples of mutation screening by heteroduplex analysis. Pedigree B505 shows the segregation of two different mutations in exon 22.

Figure 7: Homozygous mutations in the nCL1 gene

Detection by sequencing of mutations in exons 2 (a), 8 (b), 13 (c) and 22 (d). Sequences from a healthy control are shown above each mutant sequence. Asterisks indicate the position of the mutated nucleotides. The consequences on codon and amino acid residues are indicated on the left of the figure together with the name of the family.

Figure 8 : Structure of nCL1 gene

Figure 8A represents the 5' part of the gene with exon 1.

Figure 8B represents the part of the gene including exons 2 to 8,

Figure 8C represents the part of the gene including exon 9,

Figure 8D represents the part of the gene including exons 10 to 24 including the 3' non transcribed region.

EXAMPLES

EXAMPLE 1

Localisation of the nCL1 within the LGMD2A interval

Detailed genetic and physical maps of the LGMD2A region were constructed (Fougerousse et al., 1994), following the primary linkage assignment to 15q (Beckmann et al., 1991). The disease locus was bracketed between the D15S129 and D15S143 markers, defining the cytogenetic boundaries of the LGMD2A region as 15q15.1-15q21.1 (Fougerousse et al., 1994). Construction and analysis of a 10-12 Mb YAC contig (Fougerousse et al., 1994) permitted us to map 33 polymorphic markers within this interval and to further narrow the LGMD2A region to between D15S514 and D15S222.

The nCL1 gene had been localised to chromosome 15 by hybridisation with sorted chromosomes and by Southern hybridisation to DNA from human-mouse cell hybrids (Ohno et al., 1989).cDNA capture using YACs from the LGMD2A interval allowed the identification of thirteen positional candidate genes. nCL1 was one of the two transcripts identified that showed muscle-specific expression as evidenced by northen blot analysis.The localisation was further confirmed by STS (for Sequence Tagged Site) assays. Primers used for the localisation of the nCL1 gene are P94in2, P94in13 and pcr6a3, as shown in Figure 1 and their characteristics being defined in Table 1.

Table 1: PCR primers used for localisation of the nCL1 gene.

Primer name	Primer sequence (5'-3')	Position within the cDNA	Annealing temp (°C)	PCR product size on	
				cDNA	genomic DNA
P94in2	ATGGAGCCAACAGAACTGA C GTATGACTCGGAAAAGAAG GT	341-360 428-448	58	108	1758
P94in13	TAAGCAAAAGCAGTCCCCA C TTGCTGTTCCTCACTTTCCT G	1893-1912 1936-1956	58	64	1043
P94-6a3	GTTTCATCTGCTGCTTCGTT CTGGTTCAGGCATACATGG T	2342-2361 2452-2471	56	130	818
P94ex1ter	TTCTTTATGTGGACCCTGAG TT ACGAACTGGATGGGGAACT	218-239 275-293	55	76	76

These primers are designed from different parts of the published human cDNA sequence (Sorimachi et al., 1989), and were used for an STS content screening on DNA from three chromosome 15 somatic cell hybrids and YACs from the LGMD2A contig. The results positioned the gene in a region previously defined as 15q15.1-q21.1 and on 3 YACs (774G4, 926G10, 923G7) localised in this region. The relative positions of STSs along the LGMD2A contig allowed to localise the gene between D15S512 and D15S488, in a candidate region suggested by linkage disequilibrium studies.

The same primers as above were used to screen a cosmid library from YAC 774G4. A group of 5 cosmids was identified (Fig. 1). Experiments with another nCL1 primer pair (P94ex1ter; Table 1) established that these cosmids cover all nCL1 exons except number 1, and that a second group of 4 cosmids contain this

exon (Fig. 1). A minimal set of three overlapping cosmids (2G8-2B11-1F11) covers the entire gene (Figure 1). DNA from these cosmids was used to construct an *EcoR*I restriction map of this region (Figure 1B).

EXAMPLE 2

Determination of the nCL1 gene sequence

Most of the sequences were obtained through shotgun sequencing of partial digests of cosmid 1F11 subcloned in M13 and bluescript vectors, and by walking with internal primers. The sequence assembly was made using the XBAP software of the Staden package (Staden) and was in agreement with the restriction map of the cosmids. Sequences of exon 1 and adjacent regions were obtained by sequencing cosmid DNA or PCR products from human genomic DNA. The first intron is still not fully sequenced, but there is evidence that it may be between 10 to 16 kb in length (based on hybridisation of restriction fragments; data not shown). The entire gene, including its 5' and 3' regions, is more than 40 kb long, and shown in Figure 8.

a) the cDNA sequence

The used technology allows the implementation of the published human cDNA sequence of nCL1 (Sorimachi 1989). It contains the missing 129 bases corresponding to the N-terminal 43 amino acids (Figure 2). It also differs from it at 12 positions. Three of which occur at third base positions of codons and preserve the encoded amino acid sequence. The other 9 differences lead to changes in amino-acid composition (Figure 2). As these different exons were sequenced repeatedly on at least 10 distinct genomes, we are confident that the sequence of Fig. 2 represents an authentic sequence and does not contain minor polymorphic variants. Furthermore, these modifications increase the local similarity with the rat nCL1 amino acid sequence (Sorimachi), although the overall similarity is still 94 %.

The ATG numbered 1 in Figure 2 is the translation initiation site based on homology with the rat nCL1, and is within a sequence with only 5 nucleotides out of 8 in common with the Kosak consensus sequence (Kosak M, 1984). Putative CCAAT and TATA boxes were observed 590, 324, (CCAAT) and 544 or 33 bp (TATA) upstream of the initiating ATG codon, respectively (Bucher, 1990). A GC-box binding the Sp1 protein (Dynan et al., 1983) was identified at position -477.

Consensus sequences corresponding to potential muscle-specific regulatory elements were identified (Fig. 2). These include a myocyte-specific enhancer-binding factor 2 (MEF2) binding site (Cserjesi P. 1991), a CArG box (Minty A. 1986) and 6 E-boxes (binding sites for basic Helix-Loop-Helix proteins frequently found in members of MyoD family; Blackwell et Weintraub, 1990). The functional significance of these putative transcription factor binding sites in the regulation of nCL1 gene expression remains to be established.

Two potential AAUAAA polyadenylation signals, were identified 520 and 777 bp downstream of the TGA stop codon. The sequencing of a partial nCL1 cDNA containing a polyA tail, demonstrated that the first AAUAAA is the polyadenylation signal. The latter is embedded in a region well conserved with the rat nCL1 sequence and is followed after 4 bp by a G/T cluster, present in most genes 3' of the polyadenylation site (Birnstiel et al., 1985). The 3'-untranslated region of the nCL1 mRNA is 565 bp long. The predicted length of the cDNA should therefore be approximately 3550 or 3000 bp.

b) Comparison with calpain

The sequence of the human nCL1 gene was compared to those of other calpains thereof (Figure 3). The most telling comparisons are with the homologous rat (Accession no J05121), bovine (Accession no U07858) and porcine (Accession no U05678) sequences. The accession numbers refers to those or international genebanks, such as GeneBank (N.I.H.) or EMBL Database (EMBL, Heidelberg). High local similarities between the human and rat DNA sequences are even observed in the 5' (75%) or in different parts of the 3' untranslated regions (over 60%) (data not shown). The high extent of sequence homology manifested by the human and rat nCL1 gene in their untranslated regions is suggestive of evolutionary pressures on common putative regulatory sequences.

c) Genomic organisation of the nCL1 gene

A comparison of the published nCL1 human cDNA (Sorimachi et al., 1989) with the corresponding genomic sequence led to the identification of 24 exons ranging in length from 12 bp (exon 13) to 309 bp (exon 1), with a mean size of 100 bp (Figure 1). The size of introns ranges from 86 bp to about 10-16 kb for intron 1.

The intron-exon boundaries as shown in Table 2 exhibit close adherence to 5' and 3' splice site consensus sequences (Shapiro and Senapathy, 1987).

Table 2: Sequences at the intron-exon junctions. A score expressing adherence to the consensus was calculated for each site according to Shapiro and Senapathy (1987). Sequences of exons and introns are in upper and lower cases, respectively. Size of exons are given in parenthesis.

splice donor site	score (%)	Intron	score (%)	splice acceptor site	Exon
					Exon 1 (309 bp) ->
..CTCCGgtgagt..	88.5	<-Intron 1->	99.0	...ttttgttcacagGAAAT...	Exon 2 (70 bp) ->
..GCTAGgtagga..	83.5	<-Intron 2->	90.0	...gtgtctgcctgcagGGGAC...	Exon 3 (119 bp) ->
...TCCAGgtgagg...	92	<-Intron 3->	81.5	...acgcttctgtgcagTTCTG...	Exon 4 (134 bp) ->
..GCTAAgtaagc...	82	<-Intron 4->	81.5	...atcctctctctaagGCTCC...	Exon 5 (169 bp) ->
TTGATgtaagt..	87	<-Intron 5->	79.5	...ccatcgggcctcagGATGG...	Exon 6 (144 bp) ->
CCCGGgtgtgt.	77.5	<-Intron 6->	91	...ttactgctctacagACAAT...	Exon 7 (84 bp) ->
ATGAGgtaagc	94	<-Intron 7->	78.5	...tctgtgtgcttaagGTCCC...	Exon 8 (86 bp) ->
GATAGgtaggt	89	<-Intron 8->	91.5	...catttttcccaccagATGGA...	Exon 9 (78 bp) ->
TTCTGgtgagt.	88	<-Intron 9->	92	...ttccaacctctcagGATGT...	Exon 10 (161 bp) ->
CCCAGgtgggga..	80	<-Intron 10->	68.5	...ttctgggggtgcagATACT...	Exon 11 (170 bp) ->
...ACGAGgtgtgt...	85.5	<-Intron 11->	86	...tgtttcttctcaagGTTCC...	Exon 12 (12 bp) ->
..AAGAGgtatag...	70	<-Intron 12->	87	...tccccatctctcagATGCA...	Exon 13 (209 bp) ->
...TCTGAgtgagt...	76.5	<-Intron 13->	97	...tgtattcctcacagGGAAG...	Exon 14 (37 bp) ->
...CAGTGgtgagt...	89	<-Intron 14->	93.5	...ctttcttatgcagAAAAA...	Exon 15 (18 bp) ->
...CCAAGgtaggt...	89	<-Intron 15->	87	...cctcctctctccagCCCAT...	Exon16 (114 bp) ->
..CACAGgtgtct...	80	<-Intron 16->	88	...ttgtgcctccacagCCACA...	Exon 17 (78 bp) ->
...GAGATgtgagt...	84	<-Intron 17->	92.5	...ccctcctcctcagGACAT...	Exon 18 (58 bp) ->
.CAAACgtgagt...	83	<-Intron 18->	90	...ctccatcccccccagACAAG...	Exon 19 (65 bp) ->
..TGGATgtatcc..	56	<-Intron 19->	88	...cctcccctcctccagACAGA...	Exon 20 (69 bp) ->
...GGCAGgtgggga...	80	<-Intron 20->	94	...tttctattgccagAAATA...	Exon 21 (79 bp) ->
.CGCAGgtgctg..	66	<-Intron 21->	91	...ggtcccctccacagGATTC...	Exon 22 (117 bp) ->

...GTTCAgtaagt..	79	<-Intron 22->	93.5	...gcattctttcacagGAGCT...	Exon 23 (59 bp) ->
..TGGAGgtaaag..	81	<-Intron 23->	79	...gggactctttcagTGGCT...	Exon 24 (27 bp) ->

When the genomic sequence was submitted to GRAIL analysis (Uberbacher et al., 1991), 11 exons were correctly recognised, 4 were not identified, 6 were inadequately defined and 2 were too small to be recognised (data not shown).

As already noted, the nCL1 gene has three unique sequence blocks, NS (amino acid residues 1 to 61), IS1 (residues 267 to 329) and IS2 (residues 578 to 653). It is interesting to note that each of these sequences, as well as the nuclear translocation signal inside IS2, are essentially flanked by introns (Fig. 4). The exon-intron organisation of the human nCL1 is similar to that reported for the chicken CANP (the only other large subunit calpain gene whose genomic structure is known; (Emori et al., 1986).

Four microsatellite sequences were identified. Two of them are in the distal part of the first intron: an $(AT)_{14}$ and an previously identified mixed-pattern microsatellite, S774G4B8, which was demonstrated to be non polymorphic (Fougerousse et al., 1994). A $(TA)_7(CA)_4(GA)_{13}$ was identified in the second intron and genotyping of 64 CEPH unrelated individuals revealed two alleles (with frequencies of 0.10 and 0.90). The fourth microsatellite is a mixed $(CA)_n(TA)_m$ repeat present in the 9th intron. The latter and the $(AT)_{14}$ repeat have not been investigated for polymorphism. Fourteen repetitive sequences of the Alu family and one Mer2 repeat were identified in the nCL1 gene (Fig. 1C), which has, thus, on the average one Alu element per 2.5 kb.

Southern blot experiments (Ohno et al., 1989) and STS screening (data not shown) suggest that there is but one copy per genome of this member of the calpain family.

EXAMPLE 3

Expression of the nCL1 gene

The pattern of tissue-specificity was investigated by northern blot hybridisation with a genomic subclone probe from cosmid 1F11 spanning exons 20 and 21.There is no evidence for the existence of an alternatively spliced form of nCL1, although this cannot be excluded. A transcript of about 3.4-3.6 kb was

detected in skeletal muscle mRNA (Figure 5). This size therefore favours that the position -544 is the functional TATA box.

Transcription studies suggested that it is an active gene rather than a pseudogene and its muscle-specific pattern of expression is consistent with the phenotype of this disorder (Sorimachi et al., 1989 and Figure 5).

EXAMPLE 4

Mutation screening

nCL1 fulfils both positional and functional criteria to be a candidate gene for LGMD2A. To evaluate its role in the etiology of this disorder, nCL1 was systematically screened in 38 LGMD2 families for the presence of nucleotide changes using a combination of heteroduplex (Keen et al., 1991) and direct sequence analyses.

PCR primers were designed to specifically amplify the exons and splice junctions and also the regions containing the putative CAT, TATA boxes and the polyadenylation signal of the gene as shown in Table 3.

Table 3: PCR primers used for the analysis of the nCL1 gene in LGMD patients.

amplified region	Primer sequences (5'-3')	Size (bp)	Annealing temp. (°C)
promotor	TTCAGTACCTCCCGTTCACC GATGCTTGAGCCAGGAAAAC	296	59
exon 1	CTTTCCTTGAAGGTAGCTGTAT GAGGTGCTGAGTGAGAGGAC	438	60
exon 2	ACTCCGTCTCAAAAAAATACCT ATTGTCCCTTTACCTCCTGG	239	57
exon 3	TGGAAGTAGGAGAGTGGGCA GGGTAGATGGGTGGGAAGTT	354	58
exon 4	GAGGAATGTGGAGGAAGGAC TTCCTGTGAGTGAGGTCTCG	292	59
exon 5	GGAACTCTGTGACCCCAAAT TCCTCAAACAAAACATTCGC	325	56
exon 6	GTTCCCTACATTCTCCATCG GTTATTTCAACCCAGACCCTT	315	57
exon 7	AATGGGTTCTCTGGTTACTGC AGCACGAAAAGCAAAGATAAA	333	56
exon 8	GTAAGAGATTTGCCCCCCAG TCTGCGGATCATTGGTTTTG	321	58
exon 9	CCTTCCCTTCTTCCTGCTTC CTCTCTTCCCCACCCTTACC	173	56
exon 10	CCTCCTCACCTGCTCCCATA TTTTTCGGCTTAGACCCTCC	251	56
exon 11	TGTGGGGAATAGAAATAAATGG CCAGGAGCTCTGTGGGTCA	355	57
exon 12	GGCTCCTCATCCTCATTCACA GTGGAGGAGGGTGAGTGTGC	312	61
exon 13	TGTGGCAGGACAGGACGTTC	337	60

	TTCAACCTCTGGAGTGGGCC		
exon 14	CACCAGAGCAAACCGTCCAC	230	61
	ACAGCCCAGACTCCCATTCC		
exon 15	TTCTCTTCTCCCTTCACCCT	225	57
	ACACACTTCATGCTCTCTACCC		
exon 16	CCGCCTATTCCTTTCCTCTT	331	56
	GACAAACTCCTGGGAAGCCT		
exon 17	ACCTCTGACCCCTGTGAACC	270	61
	TGTGGATTTGTGTGCTACGC		
exon 18	CATAAATAGCACCGACAGGGA	258	59
	GGGATGGAGAAGAGTGAGGA		
exon 19	TCCTCACTCTTCTCCATCCC	159	57
	ACCCTGTATGTTGCCTTGG		
exons 20-21	GGGGATTTTGCTGTGTGCTG	333	61
	ATTCCTGCTCCCACCGTCTC		
exon 22	CACAGAGTGTCCGAGAGGCA	282	57
	GGAGATTATCAGGTGAGATGCC		
exons 22-23	CAGAGTGTCCGAGAGGCAGGG	608	61
	CGTTGACCCCTCCACCTTGA		
exon 24	GGGAAAACATGCACCTTCTT	375	58
	TAGGGGGTAAAATGGAGGAG		
polyadenylation signal	ACTAACTCAGTGGAATAGGG	413	56
	GGAGCTAGGATAGCTCAAT		

PCR products made on DNA from blood of specific LGMD2A patients were then subjected either to heteroduplex analysis or to direct sequencing, depending on whether the mutation, based on haplotype analysis, was expected to be homozygous or heterozygous, respectively. It was occasionally necessary to clone the PCR products to precisely identify the mutations (i.e., for microdeletions or insertions and for some heterozygotes). Disease-associated mutations are summarised in Table 4 hereunder and their position along the protein is shown in Fig. 4.

Table 4: nCL1 mutations in LGMD2A families.

Codons and amino acid positions are numbered on the basis of the cDNA sequence starting from ATG.

Exon	Families	Nucleotide position	Nucleotide change	Amino acid position	Amino acid change	Restriction si
2	B519*	328	CGA->TGA	110	Arg->stop	
4	M42	545	CTG -> CAG	182	Leu->Gln	
4	M1394; M2888	550	CAA -> CA	184	frameshift	
5	M35; M37	701	GGG -> GAG	234	Gly->Glu	

6	M32	945	CGG -> CG	315	frameshift	-SmaI
8	M2407*	1061	GTG -> GGG	354	Val-> Gly	
8	M1394	1079	TGG -> TAG	360	Trp->stop	-BstnI, -Eco
11	M2888	1468	CGG -> TGG	490	Arg->Trp	
13	R12*	1715	CGG -> CAG	572	Arg->Gln	-MspI
19	R27	2069-2070	deletion AC	690	frameshift	
21	R14: R17	2230	AGC -> GGC	744	Ser->Gly	-AluI
22	A*: B501*: M32	2306	CGG -> CAG	769	Arg->Gln	
22	B505	2313-2316	deletion AGAC	771-772	frameshift	
22	R14: B505	2362-2363	AG -> TCATCT	788	frameshift	

The first letter of the family code refers to the origin of the population B= Brazil, M= metropolitan France, R = Isle of La Réunion, A= Amish.

Each mutation was confirmed by heteroduplex analysis, by sequencing of both strands in several members of the family or by enzymatic digestion when the mutation resulted in the modification of a restriction site. Segregation analyses of the mutations, performed on DNAs from all available members of the families, confirmed that these sequence variations are on the parental chromosome carrying the LGMD2A mutation. To exclude the possibility that the missense substitutions might be polymorphisms, their presence was systematically tested in a control population: none of these mutations was seen among 120 control chromosomes from the CEPH reference families.

EXAMPLE 5 :

Analysis of families genes, chromosome-15 ascertained families

The initial screening for causative mutations was performed on families, each containing a LGMD gene located on chromosome 15. These included families from the Island of La Réunion (Beckmann et al., 1991), from the Old Order Amish from northern Indiana (Young et al., 1992,) and 2 Brazilian families (Passos Bueno et al., 1993).

a) Reunion Island families

Genealogical studies and geographic isolation of the families from the Isle of La Réunion were suggestive of a single founder effect. Genetic analyses are,

however, inconsistent with this hypothesis as the families present haplotype heterogeneity. At least. six different carrier chromosomes are encountered, (with affected individuals in several families being compound heterozygotes). Distinct mutations corresponding to four of these six haplotypes have been identified thus far.

In family R14, exons 13, 21 and 22 showed evidence for sequence variation upon heteroduplex analysis (Fig. 6). Sequencing of the associated PCR products revealed (i) a polymorphism in exon 13, (ii) a missense mutation (A->G) in exon 21 transforming the Ser^{744} residue to a glycine in the loop of the second EF-hand in domain IV of the protein (Figure 4), and (iii) a frameshift mutation in exon 22. The exon 21 mutation and the polymorphism in exon 13 form an haplotype which is also encountered in family R17. Subcloning of the PCR products was necessary to identify the exon 22 mutation. Sequencing of several clones revealed a replacement of AG by TCATCT (data not shown). This frameshift mutation causes premature termination at nucleotide 2400 where an in frame stop codon occurs (Figure. 4).

The affected individuals in family R12 are homozygous for all markers of the LGMD2A interval (Allamand, submitted). Sequencing of the PCR products of exon 13 revealed a G to A transition at base 1715 of the cDNA resulting in a substitution of glutamine for Arg^{572} (Figure. 7) within domain III, a residue which is highly conserved throughout all known calpains This mutation, detectable by loss of *MspI* restriction site, is present only in this family and in no other examined LGMD2A families or unrelated controls.

In family R27, heteroduplex analysis followed by sequencing of the PCR products of an affected child revealed a two base pair deletion in exon 19 (Figure. 6 and table 4). One AC out of three is missing at this position of the sequence, producing a stop codon at position 2069 of the cDNA sequence (Figure 4).

b) Amish families

As expected, due to multiple consanguineous links, the examined LGMD2A Northern Indiana Amish patients were homozygous for the haplotype on the chromosome bearing the mutant allele (Allamand, submitted). A (G->A) missense mutation was identified at nucleotide 2306 within exon 22 (Fig. 7). The

resulting codon change is CGG to CAG, transforming Arg^{769} to glutamine. This residue, which is conserved throughout all members of the calpain family in all species, is located in domain IV of the protein within the 3rd EF-hand at the helix-loop junction (ref). This mutation was encountered in a homozygous state in all patients from 12 chromosome 15-linked Amish families, in agreement with the haplotype analysis. We also screened six Southern Indiana Amish LGMD families, for which the chromosome 15 locus was excluded by linkage analyses (Allamand ESHG, submitted, ASHG 94). As expected, this nucleotide change was not present in any of the patients from these families, thus confirming the genetic heterogeneity of this disease in this genetically related isolate.

c) Brazilian families

As a result of consanguineous marriages, two Brazilian families (B501, B519) are homozygous for extended LGMD2A carrier haplotypes (data not shown). Sequencing PCR products from affected individuals of these families demonstrated that family B501 has the same exon 22 mutation found in northern Indiana Amish patients (Figure 7), but embedded in a completely different haplotype. In family B519, the patients carry a C to T transition in exon 2, replacing Arg^{328} with a TGA stop codon (Figure 7), thus leading, presumably, to a very truncated protein (Figure 4).

d) Analysis of other LGMD families

Having validated the role of the candidate gene in the chromosome 15 ascertained families, we next examined by heteroduplex analysis LGMD families for which linkage data were not informative. These included one Brazilian (B505) and 13 metropolitan French pedigrees.

Heteroduplex bands were revealed for exons 1, 3, 4, 5, 6, 8, 11, 22 of one or more patients (Figure 6). Of all sequence variants, 10 were identified as possible pathogenic mutations (5 missense, 1 nonsense and 4 frameshift mutations) and 3 as polymorphisms with no change of amino acid of the protein. All causative mutations identified are listed in Table 4 here-above. Identical mutations were uncovered in apparently unrelated families. The mutations shared by families M35 and M37, and M2888 and M1394, respectively, are likely to be the consequence of independent events since they are embedded in different marker haplotypes. In contrast, it is likely that the point mutation in exon

22 of the Amish and in the M32 kindreds corresponds to the same mutational event as both chromosomes share a common four marker haplotype (774G4A1-774G4A10-774G454D-774G4A2) around nCL1 (data not shown), possibly reflecting a common ancestor. The same holds true for the AG to TCATCT substitution mutation encountered in exon 22 in families B505 and R14. The exon 8 (T->G) transversion is present in the two carrier chromosomes of M2407, the only metropolitan family homozygous by haplotype, possibly reflecting an undocumented consanguinity. For some families, no disease-causing mutation has been detected thus far (M40 for example).

In addition to the polymorphism present in exon 13 in families R14 and R17 (position 668) and in the intragenic microsatellites, four additional neutral variations were detected: a (T->C) transition at position 96, abolishing a *Dde*I restriction site in exon 1 in M31; a (C->T) transition in exon 3 (position 495) in M40 and in M37 forming a haplotype with the exon 5 mutation (in the former family, this polymorphism does not cosegregate with the disease); a (T->C) transition in the paternally derived promotor in M42 at position -428, which was also evidenced in healthy controls; and a variable poly(G) in intron 22 close to the splice site in families R20, R11, R19, M35 and M37. The latter is also present in the members of the CEPH families, but is not useful as a genetic marker as the visualisation and interpretation of mononucleotide repeat alleles is difficult.

In total, sixteen independent mutational events representing fourteen different mutations were identified. All mutations cosegregate with the disease in LGMD2A families. The characterised morbid calpain alleles contain nucleotide changes which were not found in alleles from normal individual. The discovery of two nonsense and five frameshift mutations in nCL1 supports the hypothesis that a deficiency of this product causes LGMD2A. All seven mutations result in a premature in-frame stop codon, leading to the production of truncated and presumably inactive proteins (Figure 4). Evidences for the morbidity of the missense mutations come from (1) the relative high incidence of such mutations among LGMD2A patients ; although it is difficult in the absence of functional assays to differentiate between a polymorphism and a morbid mutation, the occurrence of different "missense" mutations in this gene cannot all be

accounted for as rare private polymorphisms: (2) the failure to observe these mutations in control chromosomes, and (3) the occurrence of mutations in evolutionarily conserved residues and/or in regions of documented functional importance. Four of seven missense mutations change an amino acid which is conserved in all known members of the calpain family in all species (Figure 3). Two of the remaining mutations affect less conserved amino acid residues, but are located in important functional domains. The substitution V354G in exon 8 is 4 residues before the asparagine at the active site and S744G in exon 21 is within the loop of the second EF-hand and may impair the calcium-dependent regulation of calpain activity or the interaction with a small subunit (Figure 4). Several missense mutations change a hydrophobic residue to a polar one, or vice versa (Table 4) possibly disrupting higher order structures.

METHODS

Description of the patients

The LGMD2A families analysed were from 4 different geographic origins. They included 3 Brazilian families, 13 interrelated nuclear families from the Isle of la Réunion, 10 French metropolitan families and 12 US Amish families. The majority of these families were previously ascertained to belong to the chromosome 15 group by linkage analysis (Beckmann, 1991; Young, Passos-Bueno et al., 1993). However, some families from metropolitan France as well as one Brazilian family, B505, had non significant lodscores for chromosome 15. Genomic DNA was obtained from peripheral blood lymphocytes.

Sequencing of cosmid c774G4-1F11 and EcoRI restriction map of cosmids.

Cosmid 1F11 (Figure 1C) was subcloned following DNA preparation through Qiagen procedure (Qiagen Inc., USA) and partial digestion with eitner *Sau*3A, *Rsa*I or *Alu*I. Size-selected restriction fragments were recovered fom low-melting agarose and eventually ligated with M13 or Bluescript (Stratagene, USA) vectors. After electroporation in *E.coli*, recombinant colonies were picked in 100 µl of LB/ampicillin media. PCR reactions were performed on 1 µl of the culture in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.01 gelatine, 200µM of each dNTP, 1 U of Taq Polymerase (Amersham) with 100 ng of each vectors primers. Amplification was initiated by 5 min denaturation at 95°C, followed by 30 cycles of 40 sec denaturation at 92°C and 30 sec annealing

at 50°C. PCR products were purified through Microcon devices (Amicon, USA) and sequenced using the dideoxy chain termination method on an ABI sequencer (Applied Biosystems, Foster City, USA). The sequences were analysed and alignments performed using the XBAP software of the Staden package, version 93.9 (Staden, 1982). Gaps between sequence contigs were filled by walking with internal primers. *EcoR*I restriction map of cosmids was performed essentially as described in Sambrook et al. (1989).

Northern Blot analysis

The probes were labelled by random priming with dCTP-(a^{32}P). Hybridisation was performed to human multiple tissue northern blots as recommended by the manufacturer (Clontech, USA).

Analysis of PCR products from LGMD2A families

One hundred ng of human DNA were used per PCR under the buffer and cycle conditions described in Fougerousse (1994) (annealing temperature shown in Table 3). Heteroduplex analysis (Keene et al., 1991) was performed by electrophoresis of ten µl of PCR products on a 1.5 mm-thick Hydrolink MDE gels (Bioprobe) at 500-600 volt for 12-15 h depending of the fragment length. Migration profile was visualised under UV after ethidium bromide staining.

For sequence analysis, the PCR products were subjected to dye-dideoxy sequencing, after purification through microcon devices (Amicon, USA). When necessary, depending on the nature of the mutations (e.g., frameshift mutation or for some heterozygotes), the PCR products were cloned using the TA cloning kit from Invitrogen (UK). One µl of product was ligated to 25 ng of vector at 12°C overnight. After electroporation into XL1-blue bacteria, several independent clones were analysed by PCR and sequenced as described above.

The invention results from the finding that the nCL1 gene when it is mutated is involved in the etiology of LGMD2A. It is exactly the contrary to what is stated in the litterature, e.g. that the disease is accompanied by the presence of a deregulated calpain. Identification of nCL1 as the defective gene in LGMD2A represents the first example of muscular dystrophy caused by mutation affecting a gene which is not a structural component of muscle tissue, in contrast with previously identified muscular dystrophies such as Duchenne and Becker (Bonilla et al., 1988), severe childhood autosomal recessive (Matsumara et al.,

1992), Fukuyama (Matsumara et al., 1993) and merosin-deficient congenital muscular dystrophies (Tomé et al., 1994).

The understanding of the LGMD2A phenotype needs to take into account the fact that there is no active nCL1 protein in several patients, a loss compatible with the recessive manifestation of this disease. Simple models in which this protease would be involved in the degradation or destabilisation of structural components of the cytoskeleton, extracellular matrix or dystrophin complex can therefore be ruled out. Furthermore, there are no signs of such alterations by immunocytogenetic studies on LGMD2 muscle biopsies (Matsumara et al., 1993; Tomé et al., 1994). Likewise, since LGMD2A myofibers are apparently not different from other dystrophic ones, it seems unlikely that this calpain plays a role in myoblast fusion, as proposed for ubiquitous calpains (Wang et al., 1989).

All the data disclosed in these examples confirm that the nCL1 gene is a major gene involved in the disease when mutated.

The fact that morbidity results from the loss of an enzymatic activity raises hopes for novel pharmaco-therapeutic prospects. The availability of transgenic models will be an invaluable tool for these investigations.

The invention is also relative to the use of a nucleic acid or a sequence of nucleic acid of the invention, or to the use of a protein coded by the nucleic acid for the manufacturing of a drug in the prevention or treatment of LGMD2.

The finding that a defective calpain underlies the pathogenesis of LGMD2A may prove useful for the identification of the other loci involved in the LGMDs. Other forms of LGMD may indeed be caused by mutations in genes whose products are the CANP substrates or in genes involved in the regulation of nCL1 expression. Techniques such as the two-hybrid selection system (Fields et al., 1989) could lend themselves to the isolation of the natural protein substrate(s) of this calpain, and thus potentially help to identify other LGMD loci.

The invention also relates to the use of all or a part of the peptidic sequence of the enzyme, or of the enzyme, product of nCL1 gene, for the screening of the ligands of this enzyme, which might be also involved in the etiology and the morbidity of LGMD2

The ligands which might be involved are for example substrate(s), activators or inhibitors of the enzyme.

The nucleic acids of the invention might also be used in a screening method for the determination of the components which may act on the regulation of the gene expression.

A process of screening using either the enzyme or a host recombinant cell, containing the nCL1 gene and expressing the enzyme, is also a part of the invention.

The pharmacological methods, and the use of nucleic acid and peptidic sequences of the invention are very potent applications.

The methods used for such screenings of ligands or regulatory elements are those described for example for the screening of ligands using cloned receptors.

The identification of mutations in the nCL1 gene provides the means for direct prenatal or presymptomatic diagnosis and carrier detection in families in which both mutations have been identified. Gene-based accurate classification of LGMD2A families should prove useful for the differential diagnosis of this disorder.

The invention relates to a method of detection of a predisposition to LGMD2 in a family or a human being, such method comprising the steps of :

- selecting one or more exons or flanking sequences which are sensitive in said family,

- selecting the primers specific for the or these exons or their flanking sequences, a specific example being the PCR primers of Table 3, or an hybrid thereof,

- amplifying the nucleic acid sequence, the substrate for this amplification being the DNA of the human being to be checked for the predisposition, and

- comparing the amplified sequence to the corresponding sequence derived from Figure 2 or Figure 8.

Table 2 indicates the sequences of the introns-exons junctions, and primers comprising in their structure these junctions are also included in the invention.

All other primers suitable for such RNA or DNA amplification may be used in the method of the invention.

In the same way, any suitable amplification method : PCR (for Polymerase Chain Reaction ®) NASBA ® (for Nucleic acid Sequence Based Amplification), or others might be used.

The methods usually used in the detection of one site mutations, like ASO (Allele specific PCR), LCR, or ARMS (Amplification Refactory Mutation System) may be implemented with the specific primers of the invention.

The primers, such as described in Tables 1 and 3, or including junctions of Table 2, or more generally including the flanking sequences of one of the 24 exons are also a part of the invention.

The kit for the detection of a predisposition to LGMD2 by nucleic acid amplification is also in the scope of the invention, such a kit comprises a least PCR primers selected from the group of :

a) in those described in table 1

b) in those described in table 3

c) those including the introns-exons junctions of Table 2.

d) derived from primers defined in a),b) or c).

The nucleic acid sequence of claim 1 to 3 might be inserted in a viral or a retroviral vector, said vector being able to transfect a packaging cell line.

The packaging transfected cell line, might be used as a drug for gene therapy of LGMD2.

The treatment of LGMD2 disease by gene therapy is implemented by a pharmaceutical composition containing a component selected from the group of :

a) a nucleic acid sequence according to claims 1 to 4,

b) a cell line according to claim 24,

c) an aminoacid sequence according to claims 5 to 9.

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CLAIMS

1. A nucleic acid sequence comprising :

1) the sequence represented in Figure 8; or

2) the sequence represented in Figure 2; or

3) a part of the sequence of Figure 2 with the proviso that it is able to code for a protein having a calcium dependant protease activity involved in a LGMD2 disease ; or

4) a sequence derived from a sequence defined in 1), 2) or 3) by substitution, deletion or addition of one or more nucleotides with the proviso that said sequence still codes for said protease.

2. A nucleic acid sequence that is complementary to a nucleic acid sequence according to claim 1.

3. A nucleic acid sequence comprising in its structure a nucleotidic sequence according to claim 1 or 2, under the control of regulatory elements, and involved in the expression of calpaïn activity in a LGMD2 disease.

4. A nucleic acid sequence encoding the aminoacid sequence represented in Figure 2.

5. An amino acid sequence which is coded by a nucleic acid sequence according to claims 1 to 4, characterized in that it is a calcium dependent protease enzyme belonging to the calpaïn family, involved in the etiology of LGMD2.

6. An aminoacid sequence according to claim 5 or 6, characterized in that either it contains the sequence such as represented in Figure 2, or the amino acid sequence of Figure 2 modified by deletion, insertion and/or replacement of one or more amino acids with the proviso that such aminoacid sequence has the calpaïn activity involved in LGMD2 disease.

7. An amino acid sequence according to claim 5 or 6, characterized in that LGMD2 is LGMD2A.

8. A host cell unable to express a calpaïn enzyme activity, characterized in that it is transformed or transfected with a nucleic acid sequence comprising all or part of the nucleic acid sequence according to any one of claims 1 to 4.

9. Use of a nucleic acid according to one of claims 1 to 4 or a host cell according to claim 8 in the manufacturing of a drug for the prevention or the treatment of an LGMD2 disease.

10. Use of an amino acid sequence according to claims 5 to 6 in the manufacturing of a drug for the prevention or the treatment of an LGMD2 disease.

11. Use according to claims 10 or 11, characterized in that LGMD2 is LGMD2A.

12. Use of an amino acid sequence according to claims 5 to 7 for the screening of the ligands of said amino acid sequence, said ligand being selected in a group consisting of substrate(s), co-factors or regulatory components.

13. Use of a nucleic acid sequence according to one of claims 1 to 4 in a screening method for the determination of the components which may act on the regulation of gene expression of calpaïn.

14. Use of an host cell according to claim 8 in a screening method for the determination of components active on the expression of the calpaïn.

15. A method for detecting of a predisposition to a LGMD2 disease in a family or a human being, such method comprising the steps of :

- selecting one or more exons or their flanking sequences of the gene,
- selecting primers specific for these exons, or their flanking sequences, or an hybrid thereof,
- amplifying the nucleic acid sequences with these primers, the substrate for this amplification being the DNA of a human being; and
- comparing the amplified sequence to the corresponding sequence derived from Figure 2 or Figure 8.

16. The method according to claim 15, characterized in that the primers are those selected from the group of :

a) those described in Table 1;

b) those described in Table 3; and

c) those including the introns-exons junctions of Table 2;

d) those derived from the primers in a), b), or c).

17. The method according to claim 15 or 16, characterized in that LGMD2 is LGMD2A.

18. A kit for the detection of a predisposition to LGMD2 by nucleic and amplification characterized in that it comprises primers selected from the group of :

a) those described in Table 1;

b) those described in Table 3; and

c) those including the introns-exons junctions of Table 2;

d) those derived from the primers in a), b) or c).

19. Use of a host cell according to claim 8 in a manufacturing of a drug for gene therapy of an LGMD2 disease.

20. Pharmaceutical composition for the treatment of an LGMD2 disease characterized in that in contains a component selected from the group of :

a) a nucleic acid sequence according to claims 1 to 4,

b) a host cell according to claim 8,

c) an aminoacid sequence according to claims 5 to 7.

B96R&TD

Attorney's Docket No. 960629

Applicant or Patentee: BECKMANN Jacques & RICHARD Isabelle

Serial or Patent No.: 0 /

Filed or Issued:

For: LGMD GENE CODING FOR A CALCIUM DEPENDENT PROTEASE.

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c))—SMALL BUSINESS CONCERN

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
- ☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN ASSOCIATION FRANCAISE CONTRE LES MYOPATHIES

ADDRESS OF CONCERN 13 place de Rungis - F - 75013 PARIS (France)

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed, to and remain with the small business concern identified above with regard to the invention, entitled

LGMD GENE CODING FOR A CALCIUM DEPENDENT PROTEASE.

by inventor(s) BECKMANN Jacques

RICHARD Isabelle

described in

- ☐ the specification filed herewith.
- ☐ application serial no. 0 / , filed .
- ☐ patent no. , issued .

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: *Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).*

NAME _____

ADDRESS _____

_____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME _____

ADDRESS _____

_____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING _____

TITLE OF PERSON OTHER THAN OWNER _____

ADDRESS OF PERSON SIGNING _____

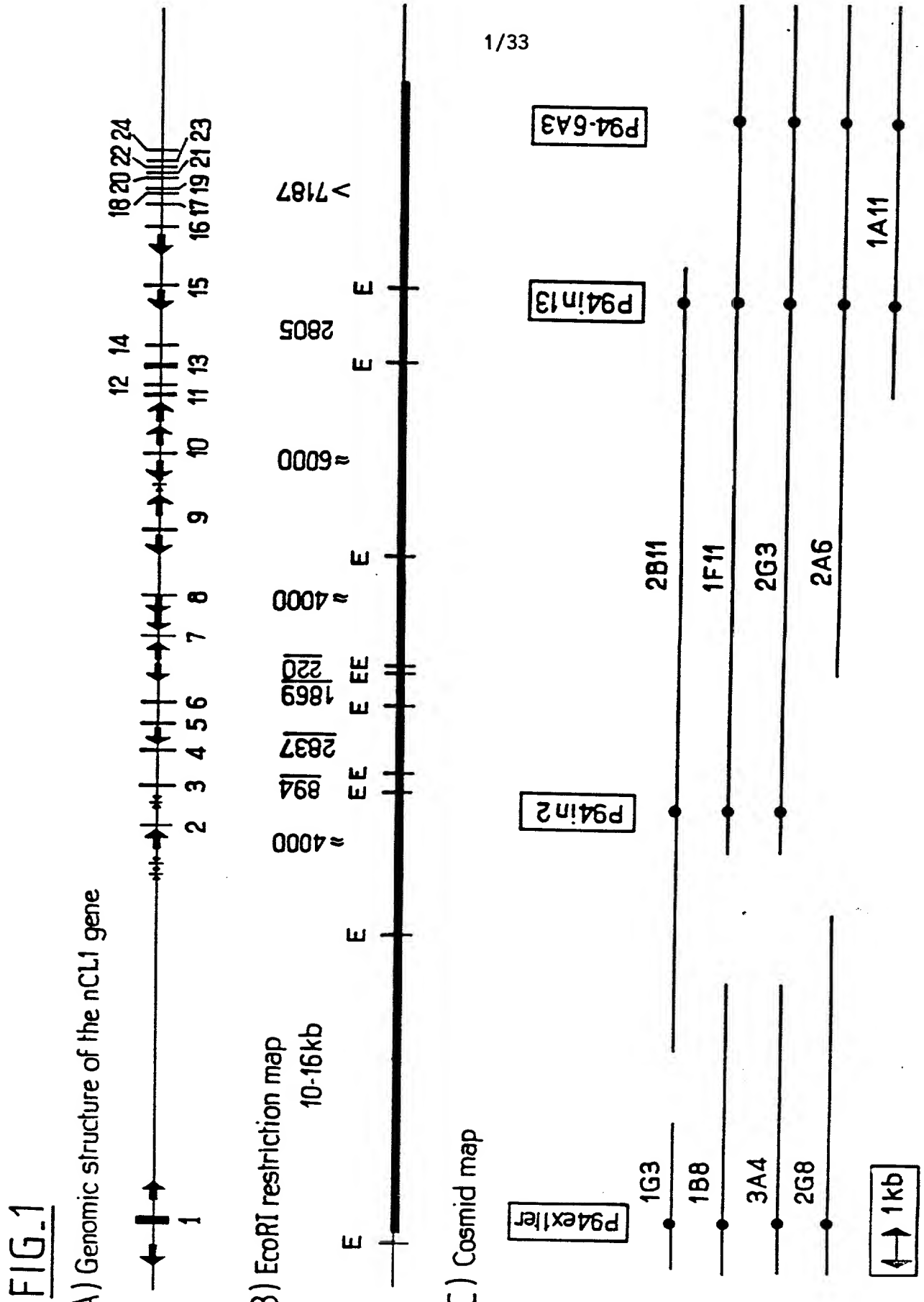
_____

SIGNATURE _____ Date le 27.05.97

Michel PIGNOLET

Vice-Président.

FIG.1



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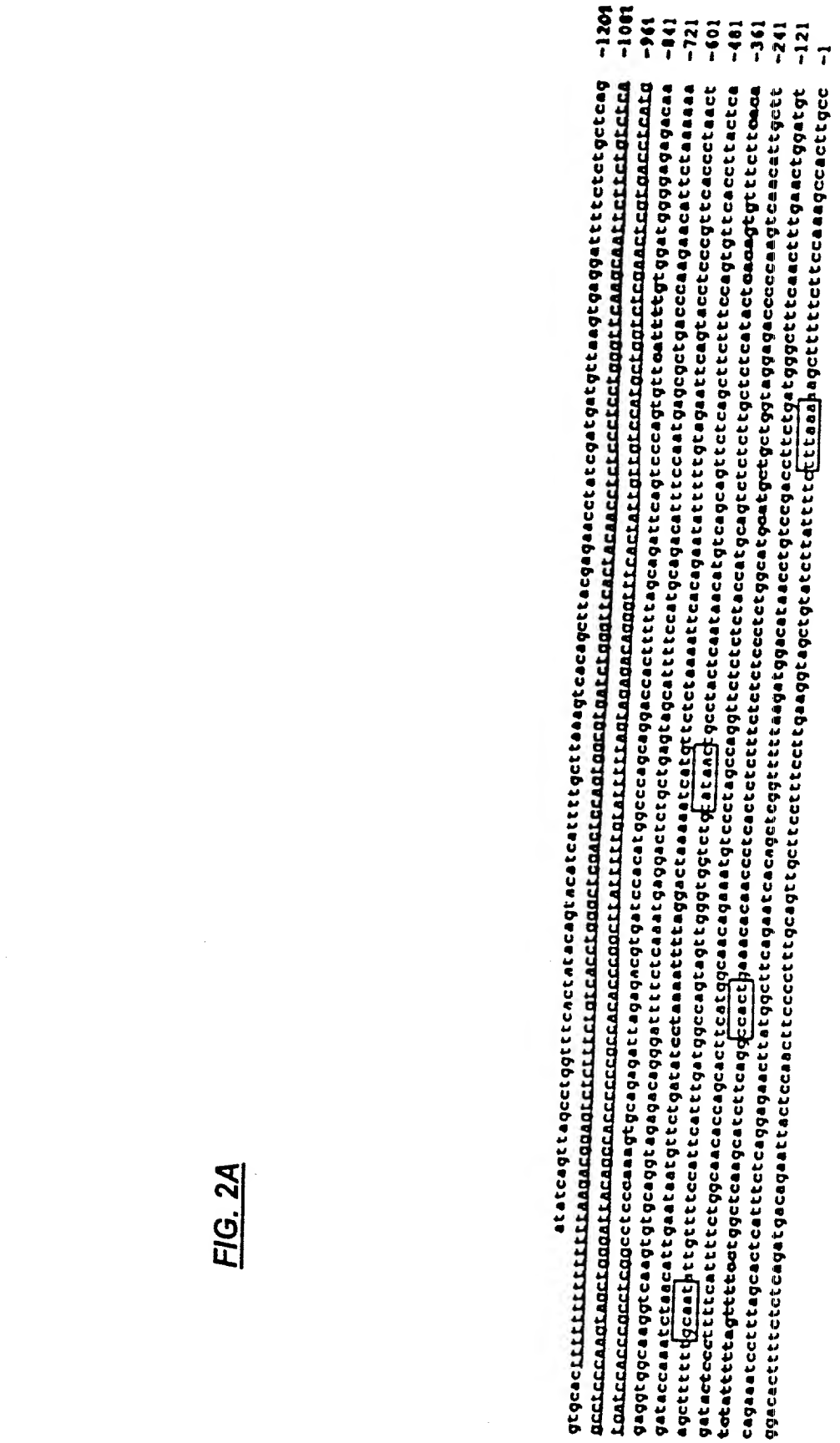
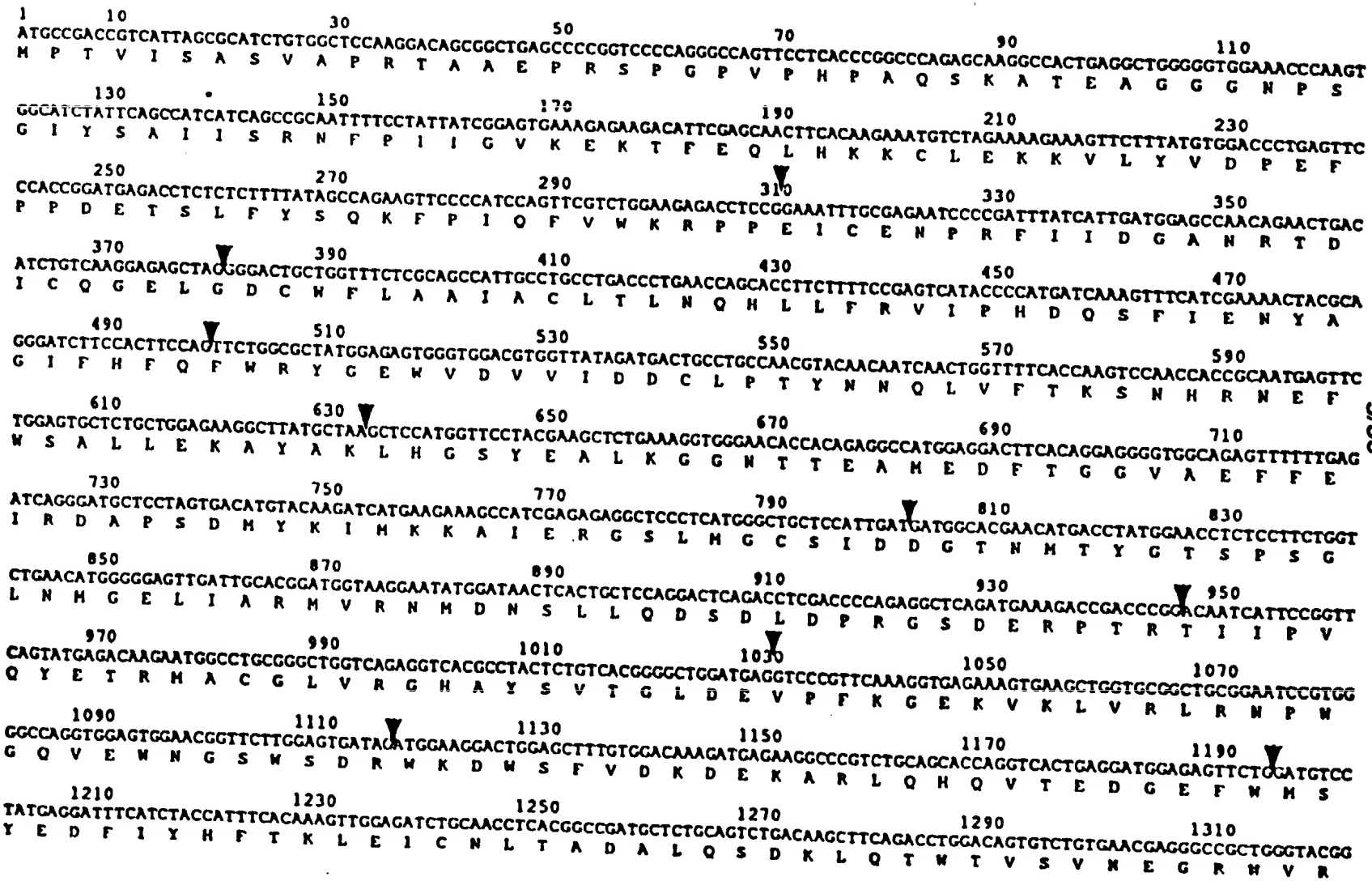


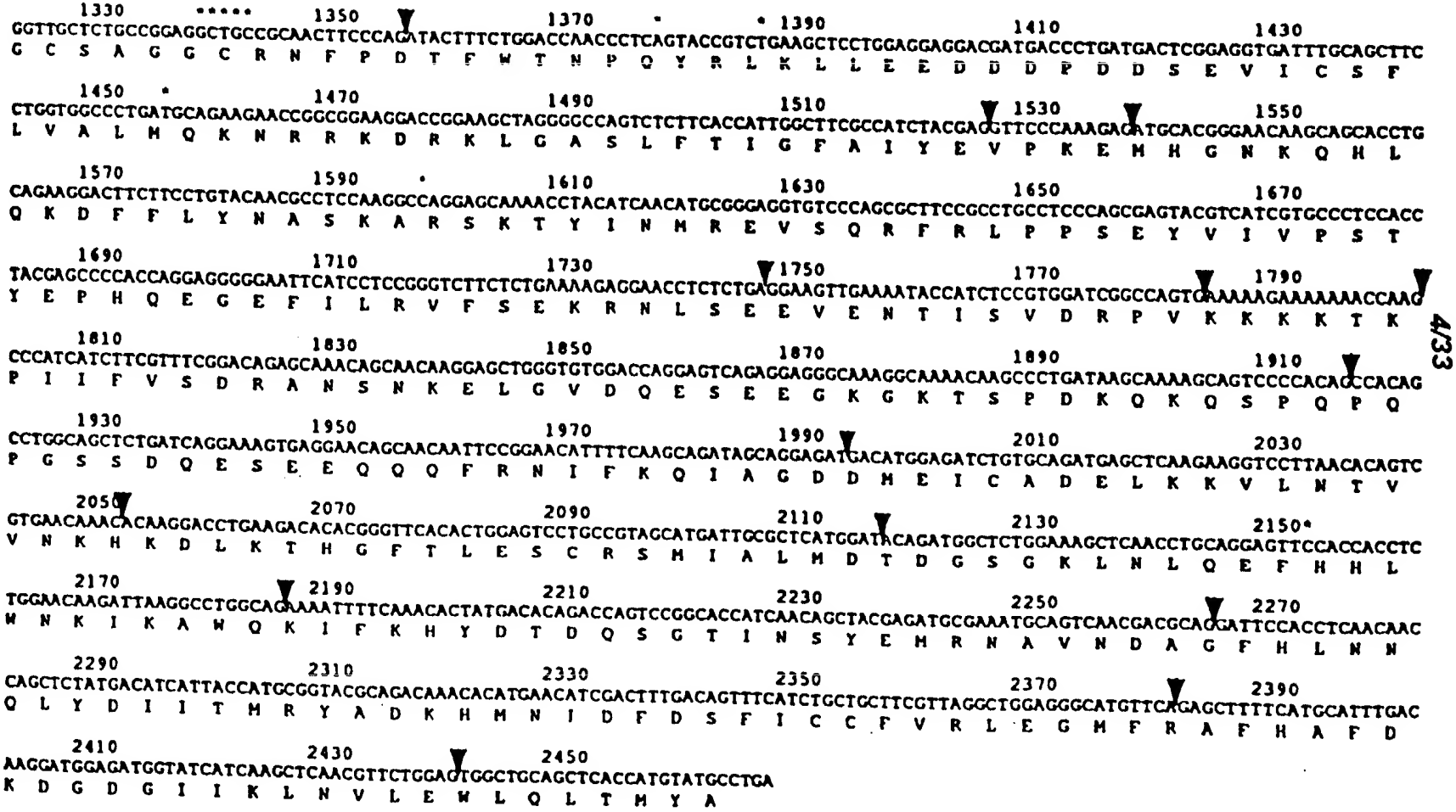
FIG. 2A

FIG. 2B/1



SUBSTITUTE SHEET (RULE 26)

FIG. 2B/2



PCT/EP95/04575

FIG. 2C

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Figure 3:

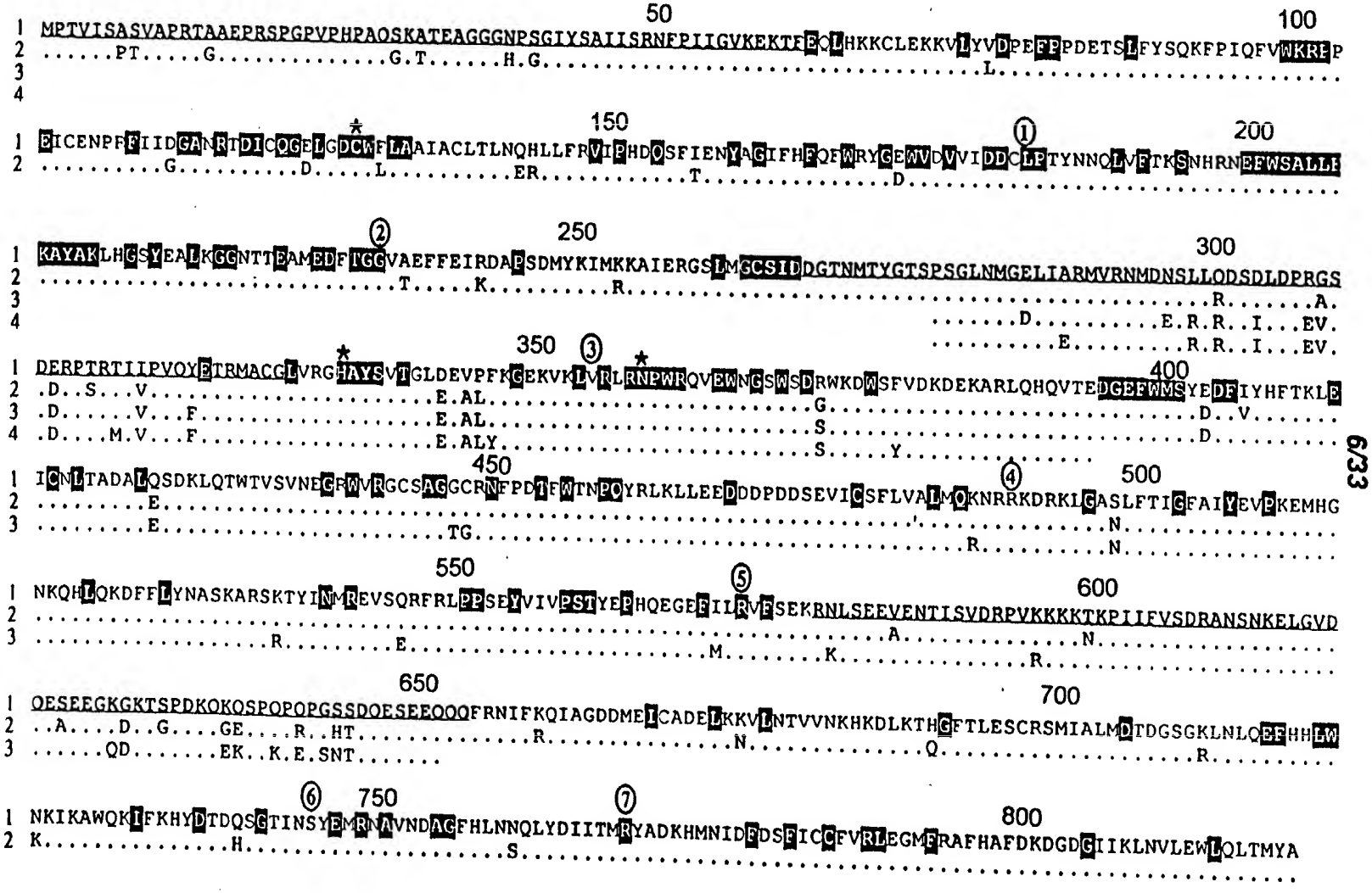
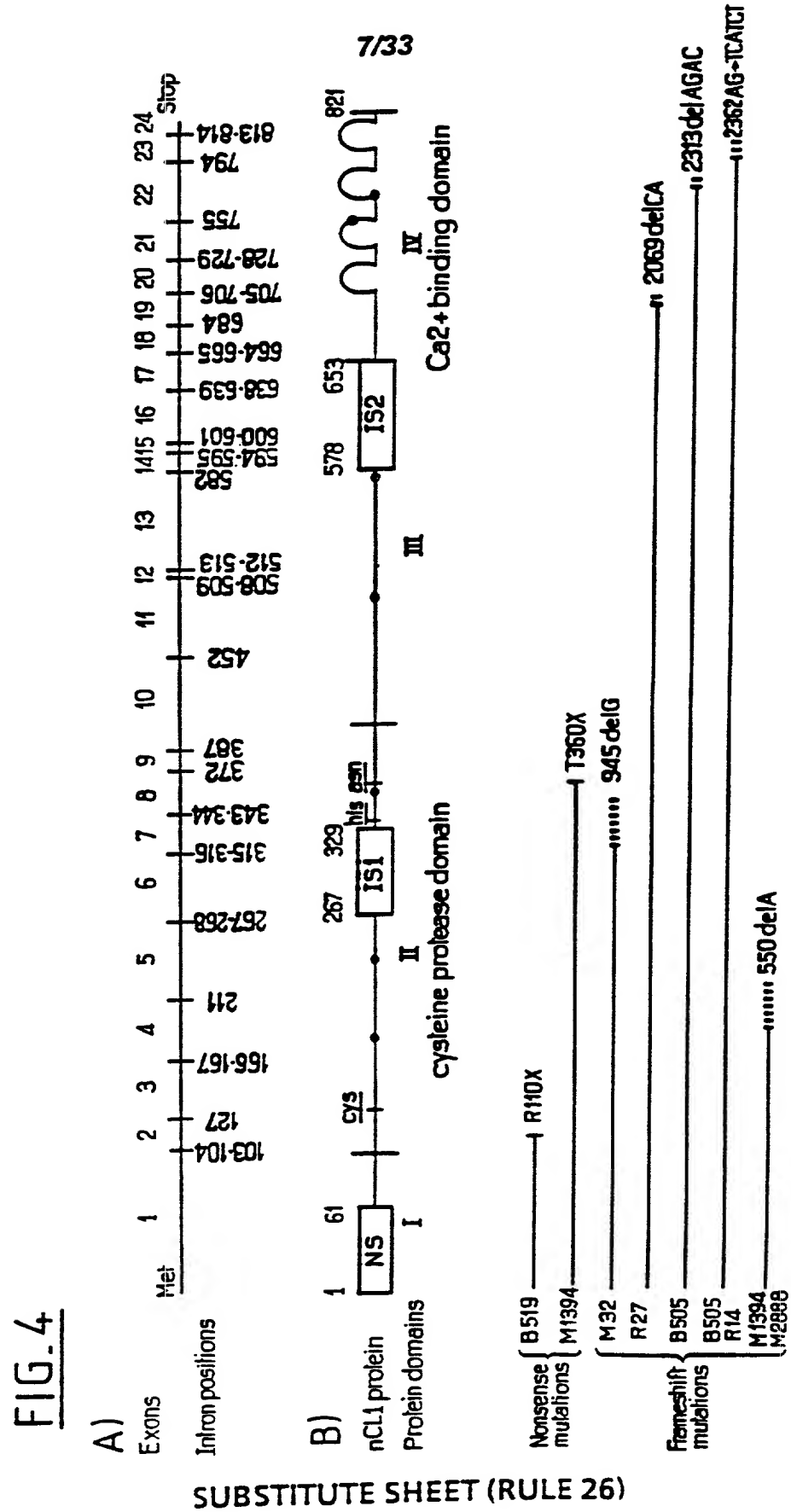


FIG.4



SUBSTITUTE SHEET (RULE 26)


heart
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kidney
pancreas
3.6 kb -

FIG. 5

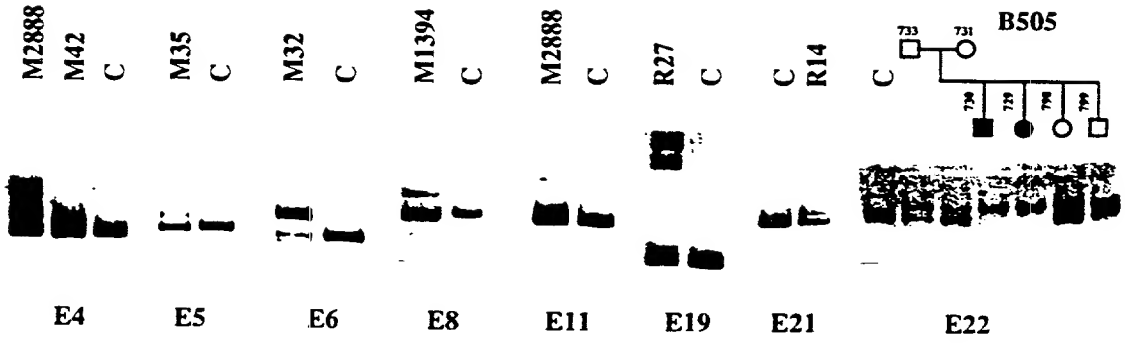
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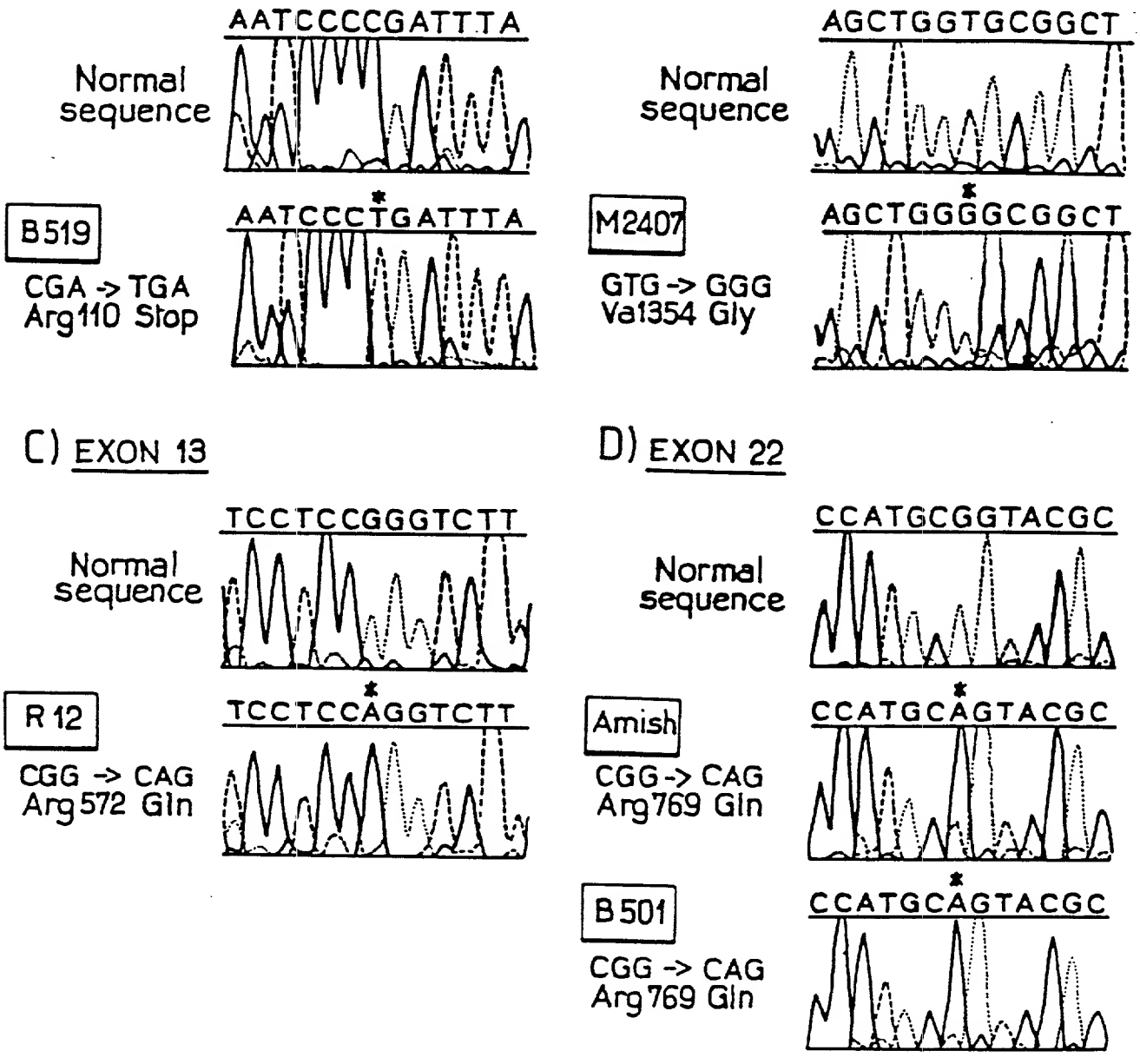
FIG. 6

FIG_7



LISTE DE SEQUENCES

(1) INFORMATION GENERALE:

(i) DEPOSANT:
(A) NOM: AFM
(B) RUE: 13, place de Rungis
(C) VILLE: PARIS
(E) PAYS: FRANCE
(F) CODE POSTAL: 75013
(G) TELEPHONE: (1) 45 65 13 00

(ii) TITRE DE L' INVENTION: LGMD GENE

(iii) NOMBRE DE SEQUENCES: 4

(iv) FORME LISIBLE PAR ORDINATEUR:
(A) TYPE DE SUPPORT: Floppy disk
(B) ORDINATEUR: IBM PC compatible
(C) SYSTEME D' EXPLOITATION: PC-DOS/MS-DOS
(D) LOGICIEL: PatentIn Release #1.0, Version #1.25 (OEB)

(2) INFORMATION POUR LA SEQ ID NO: 1:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 3018 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: double
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 1:

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GGGTTTCACT ATTGTTGACC ATGCTGGTCT CGAACTCGTG ACCTCATGTG ATCCACCCGC 420

CTCGGCCTCC CAAAGTGCAG AGATTAGAGA CGTGAGCCAC ATGGCCCAGC AGGACCACTT 480

FIG 8A/1

TTTAGCAGAT TCAGTCCCAG TGTTCATTTT GTGGATGGGG AGAGACAAGA GGTGCAAGGT 540

CAAGTGTGCA GGTAGAGACA GGGATTTTCT CAAATGAGGA CTCTGCTGAG TAGCATTTTC 600

CATGCAGACA TTTCCAATGA GCGCTGACCC AAGAACATTC TAAAAAGATA CCAAATCTAA 660

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CTCTGTCTTT AAGTGTGAAG CAGGGAGGAG AGGAACAGGT CTCAGATATT TCACCAAATC 1860

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CATCCTGTAA AAATAAATAT TCCTTTCTCA GAACAAATTC CAGACAGCCC AGATGTACCT 2040

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FIG 8A/2

```
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FIG 8A/3

(2) INFORMATION POUR LA SEQ ID NO: 2:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 11451 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: double
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 2:

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CCGACACTGC CCTAACTCTC AAGTTGCATC CTTACTCGAA TAGTATGACA GTGTGGGAAG 120

CAGCATGGGA CAATGTAAAA AGGAGGCATG TTTCTGGCTT CTGCTACTTA CTAGCTGTGT 180

GTCTTTGCAC GAGTTTCTTA ACCTCTCTGG GCCTCAGTTT CCTTATCTGA AAAATAACAA 240

TGATAGTATT CCCTTCACAG GGCCAAATGG AATACTATCA GGAACACTAC ATAATGGAAC 300

TCAATAAATA ATAGCTACTG CGGCCGGGCG CGGTGGCTCA CATCTGTAAT CCCAGCACTT 360

TGGGAGGCCG AGGCGGGTGG ATCACAAGGT CAAGAGATGG AGACCATCCT GGCCAACATG 420

GTGAAACCGT ATCTCTACTA AAGATACAAA AATTAGCTGG GCATGGTGGC GCATGCCTAT 480

AGTCCCAGCT ACTCGAGAGG CTGAGGCAGG AGAATCACTT GAACCCCGGA GGCAGAGGTT 540

TCAGTGAGCC AAGATTGCAC CAGTGCACTG CAGCCTGGCG ACAGAGTGAG ACTCCGTCTC 600

AAAAAAATAC CTATCTATCT ATCTGTCTAT CTACTGTTAT TCTTACCTGG TCATTTCCTT 660

TTTGTTTCAC AGGAAATTTG CGAGAATCCC CGATTTATCA TTGATGGAGC CAACAGAACT 720

GACATCTGTC AAGGAGAGCT AGGTAGGAAA GTGCCTCAGG TCAGATCCTG CCAGATGATC 780

AAGGGGTGAT TACAAGGTGT GATCCCCTTC CAGGAGGTAA AGGGACAATC TGTGCTTGCT 840

TCCAGTAACT TTTTGGAAGA TTTTTTATAA CAGTTGCTTT ATGGTCGTTT ATCTACATGC 900

TGGCGATTGC TTCATTTCCT CCTACATGCC TCTTTAGCAC TCTGCCATGC ATCACAGGGG 960

GTATCTGCAT CCTGTGGCCT CCTCTCCAGT ATCTCAAGGA CACTTACATA CCCCACTCAG 1020

CATGACAAAA GCCCTGCTTT TCACTGTATC GTCTTTCTTG GAAGACAGCT CTGTGACTGT 1080

GCACCAAGCA TGCCCCTTGG GCATGGAGAT TCTAGATACA CACACAAAAG GCATCGCCAA 1140

GGAAAGCACT TGTAACTGGA ACCCTTGGTT TAAATTGGCC CAGCATAGCT CCATCTTTAA 1200

FIG. 8/B I

AAGAGTCTTT CCACAAAGAT GGCATCCGCC ATGTGGATGA GCATCCAATT TTCTCTTTGA 1260

TTGGTTAGCT TGACTGCTCC ATCTGATCTT CCTCTCTCTC GACCTCTTGT TCAGAAAGTA 1320

TTGTCTTTGG TGTGGACTAT AAGCAAGCTC TGTGAAGTAA AATTGGAGAG AACACCAACA 1380

GAAACAATTT AAATTTGAGG AAAAGGGGGC ACCTAAGACC AAAGGAATTT GGCTTATTTC 1440

ATTCCAGAAG GGGAGGCTGA GAATAAATCA GATGAATATC TGGGTTCCTG CACCTGAGGG 1500

AAGGCTTCCT GCAGAGCCCT GGGCATAATA ATCTGGGACC TTCAAACCAA TAACCTCTTT 1560

TCCAAGGAAA GACTGGCTGC TTCCAAGGAG GGTAGGGGAG AGTCGGGCTG CAGGCAGCTC 1620

TCAAGTCTCC CCTTGCACAC TCTCAGGTTG GCATTTTCAC TTTAACCCAT CCTCCCTTAA 1680

GAAGGCAGTT CTTTGTGACC AGGGTACACC CCCTATTATA TATATATATA CACACACAGA 1740

GAGAGAGAGA GAGAGAGAGA GAGAGAAAGA GAGCAAAGTG TTACCTCCAA CTACATACAG 1800

TACTCTGTCA GAAAAGAGGT TCAGAGAATA AGAAAACGTC CCGAGCTCAT TCCGTTGCCA 1860

GCAATGTCTT ACTGCCCCCT ATAGACGGGT TCCAGGGCAG CTGCCTACCT GGCCTTCCTT 1920

CCAATACAAA TCATCTTGGT GGATGGTTCT CTGAGGCTCA GTCTTCGCTG AAGTCAGAAG 1980

AGGAATTGGA CTCACATTGC AAAGGCACAG GGCAGGGCAG ATTTCCTACA GGTGTTAGGA 2040

AGAACAACCC AGTTATGATC ACCTACTGCT CTGTCTCCAT TGAGGCCTAA AAAGGAAGTG 2100

AGTTTATACT GCAGTTGGAG GAACTGCCTG CAGCCTTGAG GAAAATGTCT AGTCACAAGG 2160

GAGTAAGTTA CCTGTTGATC ATATTGTCAA GGAATTCCTG TCCAATTCTC CTTCCCTGGG 2220

TTGACACCTC TGTAAGGTCA GATCTGGAAG TAGGAGAGTG GGCACCAAGG GAGTCCCCGT 2280

TCAGGGAAGT GGAGTGGCTG GCTGGGATTG GGGCTTTTTC TTCCCAGGAG GAGCAGGAGT 2340

GCTCACGATC TGTGCCCTGT GTCTGCCTGC AGGGGACTGC TGGTTTCTCG CAGCCATTGC 2400

CTGCCTGACC CTGAACCAGC ACCTTCTTTT CCGAGTCATA CCCCATGATC AAAGTTTCAT 2460

CGAAAACTAC GCAGGGATCT TCCACTTCCA GGTGAGGTAA TGAGAGTGTA GTTAAGAGGG 2520

CCAGCGGCAG GCCACCCACC GCTGGTCTCC TGGCCTTGAC TTCCCAGAAG CTGGAGGAAA 2580

CTTCCCACCC ATCTACCCGC AGCGGCAACA GTCGGCATGG ACCCCCTTAA GGCTTCAAGC 2640

CTGGGAGGAA GCAGTTGCTT ATCTCTGGCT CCCTAATCCC TCCCCCACCA CCTTCCACTA 2700

TGTCCCAGAA AGACAGGAAG ACATCCTGTT TACTGTGGGT CTATTTTTGT CTTTGCAGCT 2760

GTCTGGCTGC TTTTATTGCC TGCAGCCCTT CTCAAGTAGG TCCCTAAGAT ATTAGCACTG 2820

FIG. 8B/2

TGACACCACA GGACCCTTCA GGTTGTACAG GAACCCCTGT CCAGGGCTCC TGTATACTTC 2880

TTCCTCTCTA AGGCATCGCG GTACCAAGGC TATCACTCCT CTCTTCCAAG CCCTGGAAGA 2940

AGAGTCTGCT TAACCTGGGG ATCAGGCTTC TTGTTTGCCC TAGAACTGAA TCTGATGGTT 3000

CTAGAATCCA TCCAGCTACT GGAAATTTTC TGGGTCCCAG TCACCTTGGC ATAGAGCTGG 3060

TGCTAGAGCA GAACCAAACT GAATTCTACC TGTGAGGGTC TCGTAGCTTC CGGGATGCTG 3120

GGGAGTCAGC CTGTCTCCAG CTTCAAAGGC TCCCTCATGT CCCAGGATGA CCCACATTAT 3180

CAGTTCTTGC TCCCCGGGTC TTGCACCTCA GCACGGAAGG CCTCAGAAAA GGTCTGTCTC 3240

CAGGCTCAGA CTCCCCCTCC TGCCGCCTTG GGAACATGGC ATATTTAAAG GGTCTCAGAT 3300

CTAAAGGGCC TTACATACAA ATATCAGATA GATTTCTGTT CTCATTTCAA TGAGGGAGAA 3360

AGTGCCATTG AAAAGGAGAC TAAACCACAT TTGGCCCTTT TCAGTTCAAA CTGATTCATT 3420

CAAAAAAGAG CGACATCCAA ACTTGAAATG ATTGAACAAT GTTCCTGCTA CAGCTAGAAT 3480

AGATTCTGGG TCACTTTGTT CCTCCGTTTC AATCCTTGTT CTTCAGTTTG GCATCAAGAA 3540

ATACCTAAAT CAGCACAGTG CCTTCACTGC ATAGTTCCCA ATCCTGGCCA CATTGAATCA 3600

GCTGGGGGCA CCTGAGAGTG CTGACACCCA GGCCCTGCCC CAGACCTGCT GAGCAGGAGA 3660

ATGAAAATCT TACATCCTAA GACACTCATG GAGCACCTAC TCTACCCATT ACTGGGCTGG 3720

ACTCTGTGGA AGACATGAAG TATATGTAAC TCACTTCCAG CTCTCAAAAA GCACCCAGTC 3780

CAGTTAGAGA CAGATTTACA CACCCCAAAC ACAAAATAGG ATGAACAGGC ACCCAGATGC 3840

AGAGTCCAGG AAATGATGCT GCTTTGGGAT TCAAGAACCC CCTGAGGAAT GTGGAGGAAG 3900

GACACATTTC CTAACAGTAA TTTGAGTATG TGACTCTGTG CGTGACGCTT CTGTGCAGTT 3960

CTGGCGCTAT GGAGAGTGGG TGGACGTGGT TATAGATGAC TGCCTGCCAA CGTACAACAA 4020

TCAACTGGTT TTCACCAAGT CCAACCACCG CAATGAGTTC TGGAGTGCTC TGCTGGAGAA 4080

GGCTTATGCT AAGTAAGCAA CACTTTAGAA TGTGAGGTGG GGCTAGAGGT GAGAAAGTGG 4140

GTTGCAAAAT CCAGCCGAGA CCTCACTCAC AGGAAGAGGC ATGTGCCTCT ATACGTGCAT 4200

ATGTGTGGGC ATGCAAGTCC AACTGTGACC CAAAGTTAGA GATCAGTTCC AGGCAACAAC 4260

AGCTCTAACT AAAAACATTA AATTTAAGAG TAGAAATGAA GATTTGCATA GAAGACCTTT 4320

AGCTTTAGCT CACCATAGCG AGTTCTTTCA TTGCACCTCC ATGGTGGCAT TGCAAGTCTT 4380

GGGATCAGAG CATTGTCCCA GGGTCTCGAT TGGCTCAACC TCATGTGCTT ATAGAAGATT 4440

FIG. 8B/3

TATAAAGACA TGTTGTCTCT CAACTTAAAA GCTCCACCCC AGATGATAAT AATGGATTTT 4500

CAAATTTTGG AACAAGGTCA CTCTGTAATG CAGGCTGGAG TGCAGTGGTG CAGTCACGGA 4560

TCACTGTAGA TTGACCTCCT GGGTTCAAGG TGCTCCTCCC ACCTCAGCCT CCCAAGTAGC 4620

TGGGACTACA TGCGGGCATC ACCATGGCCC TTTTATTTTT GTATTTTTTT GTAGAGCGGG 4680

GTTTTCCCAT GTTGACCCAG ACTGTTCTCG AACTCTTGGG CTCATACAAT CCACCAGCCT 4740

TGCCCTCCCG AAGCGCTGGG ATTGCCGGTG TGAGCCACCA CACCGGCAGC TGCTAATGGC 4800

TTTAATGCAG CCCTTCCTCA ACGTTCAGGA TGTAGTGGAA AGAGCTCTCA GGAAGTGGGG 4860

ATAGCTGGGT TTCAATCCCA GTGCTTCTGG CTCTCTGTGG TCTTGGGTGG GTCACTTAGC 4920

CTCTTGAGCT CAGTTTCTTC ATTATGAAGA AAGGGAATCA TTGTTTCCAT CCCATGAGCT 4980

CATAGGGTTA ATGTGGAATT GATGAAAGAA CATCACAGCA TCCAAGAGGT AAAGTTCTGG 5040

TGGCAGTGGT ACCTGGGTTT TGTTCCCTGG AACTCTGTGA CCCCAAATTG GTCTTCATCC 5100

TCTCTCTAAG GCTCCATGGT TCCTACGAAG CTCTGAAAGG TGGGAACACC ACAGAGGCCA 5160

TGGAGGACTT CACAGGAGGG GTGGCAGAGT TTTTTGAGAT CAGGGATGCT CCTAGTGACA 5220

TGTACAAGAT CATGAAGAAA GCCATCGAGA GAGGCTCCCT CATGGGCTGC TCCATTGATG 5280

TAAGTCTGGG GTGTGGGCCA CAGGGTGGGG AGCTCCAAGT GTCAGGAAGC CTTTTACCCA 5340

ATGAAGGGCA GCATAGAGCT TTTGTGTGGG ACAGAGCGAA TGTTTTGTTT GAGGAAGCAG 5400

GAACTGGCTC TCAACTTTGA GGACTGGGAA TTTCTCAAGG GAGAACAGTT CTTCCGGATT 5460

TTCAATAAAG ACACTGGTCA AGGACATTTC AAGCCCTGGA ATGTCAGTGG AAATCAGTCC 5520

AGAGGCCTGT GTCAGTGGAG GCCTCCCTTG CTGGTGCTCC TCAGTCTCAG CACGCTCCCA 5580

TTAAGCTGGC CACGTACTTG GCTGTGGACC TGAGCCCACC ATTTCCCTAA GAAAGCCTCC 5640

CAGTCACTGG GCTTTCACCA CACCTCCCCG CTTGAGACGT GGGCTTTGTG TTGTTACCTG 5700

GGAGAAGCTA AGCCTGCAGC ACCTTTCAGT GCAAAGAAAT GCTGTGAACT GAGACAGGAG 5760

CCAAGGGTAG GGAGATGGCC GCCCATGGCC AGGCCTCCTT CAGGGGGCAT GCCTTCCCTG 5820

AGGGCTGCTC AGTATATTGA TATGATAATC TTAGTGGTTT CCATTGGGGA GGATGGGGCT 5880

GAAGCTGAAT TCCTGCCCCT TCTTCTCCCA ACACGCCCAA TGGACAGCTT GGAAGGTCAG 5940

TTAGCACACA ACACCATGGA TGAACTTTTT TTCTGTATCA CTTTTCTCCG TCTTTCCTCC 6000

ATTCGTGCTC TGTTGATCTC TCCTCTCTCC CTTTGTCTGT CCCATCTCTT TCTCCTCTCT 6060

FIG. 8B/4

CCTTCCCTTT CCACCCTTCT GTGTTTGTTC TCTCCCTCCC CTGTGTTGTT CCCTACATTC 6120

TCCATCGGGC CTCAGGATGG CACGAACATG ACCTATGGAA CCTCTCCTTC TGGTCTGAAC 6180

ATGGGGGAGT TGATTGCACG GATGGTAAGG AATATGGATA ACTCACTGCT CCAGGACTCA 6240

GACCTCGACC CCAGAGGCTC AGATGAAAGA CCGACCCGGG TGTGTACACC TCCGATTATC 6300

AGAACTGACC ATCCCTCCAA CCCACATGAC CCCGCCCTAT TAGTGTCAGA CTCCCCTCAG 6360

CAGCCAGGGC CTTACCCACA CACCCCCACC TGGCACCTCC CAAGGGTCTG GGTTGAAATA 6420

ACTTGCTCAG CCAAGGCTCC TGAAGAGGGT GCAAGAACCA GGATTTTGGA GGGAATCTCT 6480

GCTGGAGTTT CTGCATATTC CATGGTCCAG GCAGTTCCTC TCATAACGAA CTATCAGACA 6540

GAAATACTTG TAAAGATACT TCATTTATTT TGAAATATTT TTCCTCTTCT AATGTATTCA 6600

TTTATTCATT CAACACTTAT TTTTGAGCTC CTACTATGTT CCAGGCACTC CTCTAGCAAA 6660

CAAAGCAAAT TCTCTCCTCT TTTTCAATAT TTGTGGAAAA AGCAAGGTCT CCCTCTTGTA 6720

GAGTTTATAT TCTAGTATTT TCATAAGTTA TACCTGCTCA CTGGAGAATA CTGAGCCATA 6780

CAGAAAAACA CAGAGGAAAA TTTCACTTAT ATTTTTCCCC ATGTAAAGAT AACCACTCTT 6840

AACATCTAGT ATATGTTCTT CCAGGATTTT TCTATGCACA CACTGAATCT GTATTTTTAT 6900

TTTTAAAATG TTATCATATT GTATGTACCT CTTTGCAGCC TGCTTTTTTC AGTTAGTTTT 6960

TTTGGTTTTT TGGTTTTTTT TTTTTTTTGG AAACCAAGTC TTGCTCTATT CCCTAGGCTG 7020

GAGCACAGTT GTTGCCATCT CGGCTCACTG CAACCTCTGC CTCCAAAGTT AAACTAATTC 7080

TCCTGCCTCA GCCTCCCGAC ATAGCTGGGA TTACAGGCAC ACACCACCAC ACATGGCTAA 7140

TTTTTGTATT TTTTAGTAGA GACGGGGTTT CACCATGTTG GCTGGAATGG TCTTGAACTC 7200

CTGACCTCAA GTGATCCACC TGCCTCAGCC TCCCAAAGTG CTGGGATTAC AAGTGTAAGC 7260

CACCACACCC GGCCTAGTTT GATATTCTTA ATGTGCCCAA AGTATTCTCC TGTAACATTT 7320

TTTAATAGCT ACACAATATT CAAACACACA GATATGTTAT AATTTATTTA CCCAATACCC 7380

TATTATTGGA AAGTTGAGTT CTTTTTTTTC TTTGTTTTGT TTTGTTTTGC TACTATTCTA 7440

AAATGCTATA ACGAACATCC CAATAGATAC ATCTTTGTAT ACATCCATGG TGACTTCCAT 7500

AGGACAGATT CCCAGCAGTA GAATTGCTGG GTTGAATGAT ATGCTTAGGG TAATGACAGA 7560

AGAGTCATTT CAAGCAGCTT CCTAGGGTCT TAGAACTTAA GGATTAATGA GTCTTCCCGC 7620

CCCCTCCCAG TCTATTCAGC ATGATCTGGA TCATGAGGAC TGAGATCTGG AAGAGACTGA 7680

FIG. 8B/5

GATCTGGGAG AGGCTGAGAT ACCAAAAGCC CTGGCTCCAC CCATACCCCT CGCCCTGAAA 7740

ACAGCTCTAG GAATTCCGCG GCCTAGCAAG GCTCCGGGAA GCTCCTTTTA AAGCTGTGAC 7800

GTTAGTAGGC ACATGGACCA TAGAGACCTA TCCAGGGCTC ATGGGACTTT AGTGATCCTG 7860

CCCTTCTCCC AAGGATCCCC CATGGCTGCA ACTTGGAAAT TTCTGCAAAT GGAAGAGCTA 7920

CTCCTTAGGC ACGGTCATGT CTGAGCAGGG ATCTCCTCGG GCTTTCTTAG AATTCTCTCC 7980

CTGGGCACTG GGACTCTTGA TTTCTTGAAT ATTATGTTCC AGGTGGGTGT GGAGGAGGTG 8040

AGGGGATGTA AAGAAGGCTA GACTTGGCCA GGCGCAGTGG CTCATGCCTG TAATCCCAGC 8100

ACTTTGGGAG GCTGAGGCGG GTGGATCACC TGAGGTCAGG AGTTCGAGAC CAGCCTGGCT 8160

AACATGGTGA AACCCCGTTT CTACTAAAAA TACAAAAAAT TAGCTGAGCA TGGTGGCACG 8220

TGCCTGTAAT CCCAGCTACT CGGGAGGCTG AGGCAGGAGT ATCGCTGGAA CACGGGAGGC 8280

AGAGATTGCA GTGACCCGAG ATCGCGCCAC TGCACTCCAG CCTGGGCGAC ACAGCAAGAC 8340

TCTGTCTCAA AAAACAAAAA AGAAAGAAAA AAAGGAAAAG CTAAGACTTA CATGTGTCAC 8400

TTAACCCCTT TTCTCAAACC TCTTTCTCTT CCAGGAATAG TCAACCCCTG GATGGCTTCA 8460

GGGGAAGGGG GATCCTGAAG CCCAGGGCAG CCTCCAACTC TACCCCTTCC TCCTTTGAAG 8520

GATACTAAGG GGTCCAGAAA GGAGGGGCAG GACACTGTTA CCCACCCCAC ATCCCAGCAT 8580

CCACATTGCT CTCTGATGGT CAGGACAGAG CCTTCTCAGG GAGACCAGCC TGTCTGGAGC 8640

TGTGTCTCTT GGCACTCTTA AAGGGCCACT GAAGGTCCGT TCGTGGTCGT GAGGCACACT 8700

TTCAGGGAGC AGAGTGGTCT GTGTCTTCAC AGAGCCCGGA AAATGAACTA GTATGAACTT 8760

TGCCTCCAAG CAGCAGAACT TCTGTTCCCC CGCCCCTAAT GGGTTCTCTG GTTACTGCTC 8820

TACAGACAAT CATTCCGGTT CAGTATGAGA CAAGAATGGC CTGCGGGCTG GTCAGAGGTC 8880

ACGCCTACTC TGTCACGGGG CTGGATGAGG TAAGCCTGGT GGGGCTTGGT GGGGCAAGGG 8940

CACCCTCCTG GGTTAACCTC ATGAAGTCAG GACTTAGCTG TTGGGGCCCC TGCCCTGTCT 9000

GCAGAGCTTG CCTCCAATCA GGACATTCAG TTCAAGGTCC AAGCCACGCC TGGGAGCAGA 9060

GGGGCCTGTG AAACTGGTAG AGGTGGATCC TGCCACAGTT GGTGCACAGT TTATCTTTGC 9120

TTTTCGTGCT AAAGATGGCA ATTTTTCCAA CATTTCCAAT GAACAAATTG AAATATCACT 9180

TAACTTTGCT TTTACAAAGT TGGTTTCATG TGTTCTTGAG CTTCCTGTTC TCTCGTGTTC 9240

AGATAGCTAC AGTTGTCTCT GGGTAGCCAC GGGGACTGGT TCCAGAAGCC CCAACAGTAA 9300

FIG.8B/6

CAAAATCTGC AGATGCTCAA GTCCCTTCTG TAAAATGGAG TAGTATTTGC ATATAACCTA 9360

TGCACATCCT CCCATATACT TTAAGTCATC TCTGGATTAC TTACGATACC TAACACAATG 9420

GAAATGCTAT GTAAATAGTT ATTGCACTGC ATTGGGTTTT TTTGGTATTA TTTTCTGTTG 9480

TTGTATTATT ATTTTTTTCTT TTTTTGAATA TTTTTGATCC ACAATTGGTT ATATGCCAAA 9540

GCCATGGATA CGAGAGGCTG ACTGTTCTGT TTTGCTCCTT CTGGGACTTC TGGGTTTTCC 9600

TGGACCATGT CTGAGACAGG AACGTTGTAA GACCTGTTGC ACACAGTTGG GCAGGTTGTG 9660

CCCTGTACAG AGGGATGGGC TGAGAGGGGC AGTTGCCTGC ATCACCCATT GCAGCAGACT 9720

GGAGGGAGTC TGCTTGTTTG TAGTTCCTCA GTCAGCAGGG GCCTTTTGTC TTTCCTTCCT 9780

TTCCTTTTTT TTTTTTTTTG AGACGGAGTC TCACTCTGTT GCCCAGGCTG GAGTGTAGTG 9840

GCACAGTCTC GGCTCACTGC AATGTCCGCC TCCTGGATTC AAGCGATTTT CCTGCCTCAG 9900

CCTCCTGAGT AGCTGGGATT ACAGGCGCGT GTCACCATGC CCAGCTAATT TTTGTATTTT 9960

TAGTAGAGAT GGGGGTTTCT CCATGTTGAT CAGGCTGGTC TCGAACTCCT GACCTCGTGA 10020

TCCGCCCACC TCGGCCTCTC AAAGTGCTGG GATTACAGGC GTGAGCCACC ACGCCTGGCC 10080

AGCAGGGGCC TTTTTTCTAA TTTATATGAA GACACCTAAT TTATATGTGT TAGCAAAGCC 10140

CTCCTGTTTA TGCCTCACCT CCTCCCCCGA AGCTCATACG GCAGGATGTT CCTGAGAAAA 10200

TTGCCTCTTA GAAGATAGAG AGGAGATGCC AAGCCTAAGT TAGGCAGACT CAGGAGGATA 10260

GGTCTGACCC ACCCCCTGCC ATTCCCCAGC ACACTTGTGA TTAATCTCCT TGGCCAGAGC 10320

CAGGCAGAAC ACCCTCGCGT AAGAGATTTG CCCCCCAGCC CCGTCCCAGC CCTCAGCTAG 10380

ACAGAAGATT CCCTTTCCAG AGAGGCTGCA GAGCATGAGA GCTCTTTCTG TGTGCTTAAG 10440

GTCCCGTTCA AAGGTGAGAA AGTGAAGCTG GTGCGGCTGC GGAATCCGTG GGGCCAGGTG 10500

GAGTGGAACG GTTCTTGGAG TGATAGGTAG GTGAGGGGAC CCCACGGGAT TGGCGGTGGC 10560

GGGGAACAGG GTCCGGGACA AGGCTGTGTT GGGAACTGAG CCATGAGAGT ATTGAAGATG 10620

CTTGGTATAA AATCACCCTC AAAACCAATG ATCCGCAGAG AAGAGGGGCA CAGGTGTTGG 10680

CTCCAGGGAA GGGCCAGGAG TGGAAGCGGG GTGCTGGGGA CCCAGAGAGG TTGCTGACAA 10740

CCATTGGCTG GAAAGGAAGG ATTCCAGAAA GCGTGGGGAA GGTCCAGGCA GGAAAAGCGT 10800

ATGAATGCAG GGTTCTGGGC TAGAGAAGTG ACTTCCCTTC TTGGGGTCTT GTGTTGCCTT 10860

TCCTGTGAAA TGGGAACAGT ATTATTAGCA CTTACCTTGT GGGCTGATAT TGAGGAGTAA 10920

FIG.8B/7

```
CTGGGACTTG TTTTTGGGCA AGTGCTGAGC CATTGCTAAG ATTCCCCTTA CCCGTGCTTG   10980

TCCCTTGTAT TAAGGCACAA GGGCCCTTTG AAAAGAATTT TACCTGCTTT ATCAATTGAA   11040

AGGGATTAAG ACCTTGGGGG CCAACCCAAA ATAAACATGC GAACTTATTA TTTATAGGCT   11100

CCATGCACAC TTCGTAAAAC CTCCATGGTC CTACTGGTTC CTGATTACCT CCACTCAATG   11160

AGAGGCAATT CATTACTGAA TGAGCCATAA GCGCCTCTTA TTTCGAGAGG GGGATGGCAG   11220

GACTCAGTCG AGGAGAAGGA CCGCACCCAG GCAGCCTGGG CCCCTCGGCT CCTGTACTTA   11280

TTTACTGCTG GGTACTTCCT AGCCCAGCAT GTAATTACTG GTTCGTTCAG TCATTCGTTT   11340

AGTAAATGTT TCTTGGGCAC CTACTACATA GGAGGCACAG GTCAAGGCAC TGGGGATATT   11400

CTTTCTACCC ACCCCCTCCC TCCCTACACT GTGATTAGGG ACTGACCGAT C             11451
```


FIG. 8B/8

(2) INFORMATION POUR LA SEQ ID NO: 3:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 1834 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: double
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 3:

ATTTTTTTTT TTTTTTTTGA GACGGAGTCT CACTCTGCCA CCCAGGCTGG AGTGCAATGG 60

CGCGATCTTG GCTCACTGCA ACCTCCGCCT CCCGGGTTCA AGTGATTCTT CTGCCTTAGC 120

CTCCTGAGTA GCTGAGACTA TAGGTGCCCG CCACCACGCC CAGCTAATTT TTGTATTTTT 180

ATTAGGACGG GGTTTCACCA TATTGGCCAG GCTGGTCTCG AAATCCTGAC CTTGTGATCC 240

GCCCACCTCG GCCTCCCAAA GTGCTGGGAT TACAGGTGTG AGCCATTGCG AGCAGCCCAG 300

AACTCAATTC TTAACCTTTA AAGTATGATG AGAAGAAGGA TCAAGCCCTC ACCAGCCCAT 360

TTAAGGAGTT TAGGCTCAGT CTTGAGGATG TGAGAAGTCA TTGCTATTGG GTTTCACACT 420

GAGGTTAACA GGTGAAGTCA GCATTTTGGT AGTTCACAGC AGCTGCAACT CTTTGTATTT 480

CTCTGATACC TCCTGTCCCA ACCTACATCA GGCCTTCCCT TCTTCCTGCT TCCTTAATTC 540

CTCCATTTTC CCACCAGATG GAAGGACTGG AGCTTTGTGG ACAAAGATGA GAAGGCCCGT 600

CTGCAGCACC AGGTCACTGA GGATGGAGAG TTCTGGTGAG TCCAGAACCC AGGAAGACCC 660

AGAAGGGTAA GGGTGGGGAA GAGAGGGGAA ATCTCAGACC TCAGTCCCCA GCTAAGGTTA 720

TCAGATTCCA GCCCTTGGGA GATCTTGGCT GTGTTCTCCT CCAGCCCAAG GCCCAGCAAG 780

GATGAGGTTC TGAGAGGAGC CTTCCAGGCC ACAGGGACAA TGAGCCCAGG ACCAGGCCAA 840

CATGACATGG CTCTTGCCTC CTGTGTGCCC CTCCGCCACA CACTCTATTC CAGCCACAGG 900

CACCCTGGCC TTAGCACAAT TCTTTTCTGA GCCTAGGAAG CTCCACTTAC CCTGATCTTC 960

CAACGTCAAC CTCACCCTCT CTCAGGTTGT TTCTATTCAG GCTTCAAGTC TCAGCTTAAG 1020

GAGAATTTTC AAGTCTCAGC TTAAGGAGAG CCCCCTAAGT TCCCCGAGGA CTGGGATTAA 1080

TTTATGATGC TCATCACCCT TAAAATTGTT TGCTTAAGCC GGGCGCGGTG GCTCACGCCT 1140

GTAATCCCAG CACTTTGGGA GGCCGAGGTG AACGGATCAC GAGGTCAGGA GATCGAGAAC 1200

FIG. 8C/1

ATCTTGGCTA ACACGGTGAA ACCCTGTCTG TACTAAAAAT ACACAAAAAA AGTAGCCGGG 1260

CGTGGCAGCG TGCGCCTGTA GTCCTAGCTG CTGGGGAGGC TGAGGCAGGA GAATCACTTG 1320

AACCTGGGAG GCAGAGGTTA CAGTGAGCCC AGATTGCGCC ACTGCACTCC AGCCTGGGCG 1380

ACAAGAGAGA CTCTGTCTTG GAAAAAAAAA AAAAAATGTG GTCTTAGTTT AATGTCAAGG 1440

GAAAGGTTTT GGGTGTTTTT ATTACTTTAT TTTTTATTTA AAAACTATAA TAGAGACGGG 1500

CCTCGCTATA TTTCTCGGGC TGGTCTCAAA CTCCTGGGCT CAAGCGGTCC TCCCACCTTG 1560

GCCTCCCAAA ATGCTGGCAT GTGGGCCTGG TCAACATATG GGACCCCAAC TCTACAAAAA 1620

ATTTTAAAAT TAGCCAGATG TGGTGGCGTG TGCCTGTAGT CCCAGCTACT TGGGAGGCTG 1680

AAGCAGGGGG TCACTTGAGC CCAGGAGGTT GAGGCTGCAG TGAACTATGA TTGTCGTTCA 1740

CTTTTCTTCT GAACGTGAGA TTAAGTGTAG TCAGCAATTT GGCTTAGGAT TATTTATTCA 1800

GAATTTTTAA CCGTCACGTT GCGGCAAACC AGGT 1834

FIG. 8C/2

(2) INFORMATION POUR LA SEQ ID NO: 4:

(i) CARACTERISTIQUES DE LA SEQUENCE:
(A) LONGUEUR: 14664 paires de bases
(B) TYPE: acide nucléique
(C) NOMBRE DE BRINS: double
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 4:

AGGAGGTGGA GGTTGCAGTG AGCCAAGATC ATGCCACTGC ACTCTAGCCT GGGCAACAGA 60

GCGAGACTCT GTCTCAAAAA ATACACACAC ACACACACAC ACACACACAC ACACACACAC 120

ACACACATAT ATATACACAC ATATATATAC ACACACATAT ACACACACAC ACGTCTGTAT 180

ATATATGTGT GTGTGTATAT ATACACACAC ACACTATTCT ATATATTCTT GTAGAGCTAT 240

GTGTGTCTCC TGTGCTATTG AGCATGAGCC CTTTTTTTTT TTTTTTTTTT TTGAGACAGA 300

GTCTCACTTT GTCGCCCAGG CTGGCATACA ATGGCGCAAT ATCGGCTCAC TGCAACCTCC 360

GCCTCCTGGG TTCAAGTGAT TCTCCTGCCT CAGCCTCCCA AGTAACTAGG ATTACAAGTG 420

CCCGCCATAA TGCTCAGCTA ATTTTTGTAT TTTCAGTAGA GATGGGGTTT CACCATGTTG 480

GCCAAGCTGG TCTCAAACTC CTAGCCTCAG GTGATCCACC TGCCTCAGCC TCCCAAAGTG 540

CTGGGATTAC AGGCATGAGC CACAGCACCC TGGTGAGCAC TAGAGCTTAT TTCTTCTATC 600

TAACTGTATT TTTGTATCCA TTAGCCACCC TCTTTTCATC CTCCCCTCTC CTTCCCTTCC 660

CAGCCTCTGG TAACCACTGT CTGCTCTCTA CTTCCATGAC ATATGCTTTG TTTTAGCTCT 720

CACATATGAG TGAGAGCATG CGACATTTAT CTTTCTGGCC CTGGCACATT TTTGAATCAT 780

TGTTAGAAAA GATGATGGTT TGGAGTAGAT ACATCAGAAG TGACAGCGTT TGCCCTAAAA 840

AGGAAAGACA GGCTCCTCTG GGACCCTGAC CAAGTTCCTG TGAACTATTT TATTATTGTG 900

CTGTGTTAGT CCTGGGGTCT TCCGTTCCCA GCCCTCCTCA CCTGCTCCCA TATGGCTCTC 960

TCTCTTCTTC CAACCTCTCA GGATGTCCTA TGAGGATTTC ATCTACCATT TCACAAAGTT 1020

GGAGATCTGC AACCTCACGG CCGATGCTCT GCAGTCTGAC AAGCTTCAGA CCTGGACAGT 1080

GTCTGTGAAC GAGGGCCGCT GGGTACGGGG TTGCTCTGCC GGAGGCTGCC GCAACTTCCC 1140

AGGTGGGAGA TGCTCTTGAT GGGGGGAGGG TCTAAGCCGA AAAAGTTCCA GGCAGAAGAA 1200

FIG. 8D/1

GCCTAACTAG TGCTTATTAA GTCTCTCTGT TCCAGACGTC CACTATCTTA TTAAACCTTC 1260

CCTGTTTTAC TGAGAAGGAA ACCACCATGC TGAGAAGTTT GCAATAGGGA GCTGGGTAGC 1320

AACTTTGGAA GCAGGAACTT GTGGGAACAA TGCAGATGCT GCTTGGACTT ACGATGAGGT 1380

TATGTCCAGA TAAGCCCATC CATCTTTTGA AAATACCCTA AGTGAAAAGT GCATCCAATA 1440

TGCCTAACCC CCCAAACCTC ATAGCTTACC CTGGCCTACC CTCAAACATT GCTCGGAACC 1500

CTTGACCTTA AGCCTAAAGT TGGGCCAAAT CATCTAACTC CAAAGCCTAT TTTACAAAGA 1560

AAGTTGTTGT AATATCTCCA TGTAACTTAC TTAATACTTG TACCTAAAAA GTGAAAAACA 1620

AGAATGGTTG TACGGGTACT CGAAATCCAG TTTCTACTGA ATGTGCATCT CTTTCACATT 1680

GTAAAGTTAA AAAATTGTAG CCGAACCATC CTAAGTCAGG GACTGTGAGT ACTGTGTCAG 1740

TAACAGTAAG GGCACTATTG GAGAACCAAG TTAGCAGCTG CTGCAATAGT TCAAGTCAGA 1800

GATGATGAAA ACCTAGACCA AGTCAGTAGC AGCAGAGATG GAGGGGAGAC AGCAGATTTA 1860

GGGAGAGCAT ATTGGGTGAT GTAGGGAAGG AAGAAGAATG ATGTCAAGAT TCCCAGTTGG 1920

GGACCTGACA ACATTGCAAC ATAAGACACA CAAGAAGATC GGGTGGGTGG CTCATGCCTA 1980

TAATCCCAGC ACTTTGGGAG GCAGAGCCAG GAGGATCACT TGAGCCCAGG AGTTCAAGAC 2040

CAGCACAGGC AACATAGTGA CACCTCATCG TTACCCAAAA TAAAAAAAAA AATGAGGTGG 2100

GAGGATTGCT TGAGCTCGGG AGGTTGAGGC TACAATAAAC TGTGATCATG CCACTGCACT 2160

CCTGCCTGGG TGACAGAGTG AGACCCTGCC TCAAAAAAAA AAGACACACA AGAGAAAAAT 2220

ATCAGCGTGT TGTTTGTTTT TGGTGGAGTT AATTGTGGGG TTCTAGGGAA AGGAATTTAG 2280

CTTGGGACAT GGAAAGTTTG AGGTTCCTGT AGAGTGTCCC AGTGAAGATT TGTAATAGAG 2340

CATCGGATGC GCATATTAGA TGGCACTTGG TGATATGATA AGAACTCAAA AAATATTTGA 2400

GGAATAAAGG AAAGAAGAGG CCAGACGTGG TGGCTTATGC CTGTAATCCC AGCACTTTGG 2460

GAGGCTGAGG CAGGCGGATC ACTTGTGGTC AGGAGTTCGA GACCAGCTTG GCTAACATGG 2520

TGAAAACCCA TCTCTACTAA AGATACAAAA ATTAACCGGG GATGATGGTG GGTGCCTGTA 2580

ATCCCAGCTA CTTGGGAGGC TCAGTCAGAA GAATCGCTTG AACCCAGGAG GCGGAGGCTG 2640

CAGTGAGCCG AGATCGCGCC ACTGCACTCT AGCCTGGGCA ACAGAGCCAG ACTCCGTCTC 2700

AAAAAAAAAA AAGTGAGAGA GATTGAGGCT GGGATATATG GCTCAGGCAT CATGCGCGTG 2760

TAGGGGGCAG TTAAAAAGCA GAAGTAAGAA AGATTGCCTA GGGAGGCAGG AAGGGTGAGG 2820

FIG. 8D/2

TGAGAGGAGA AGAGGCCCAG GACCAGATTC TAGTCACCAA CAGCGTTTAA GGGGCAGGTA 2880

AGGAAAACAA AACCATCAGC AAAGACTGAG AATGAAAGCC CAGAGAGGAA GGAAAAGCCA 2940

CACATACAAT CAGTACAGCT CCATCTGAAT AAAGGTAGCG CCCCCCCCCC CCCAAATCAT 3000

TAGAGAAATG CCTGATTCGG TTTTCTGTGG ATTTTTCCTA AGAACCTAGA TGTGGGGAAT 3060

AGAAATAAAT GGTTCCCTCT GTCTCATCCC CTCCCTGCCC TCTGAGAGGA AGCTGTGATT 3120

GCGTGCTCCC TTTCTGGGGG TGCAGATACT TTCTGGACCA ACCCTCAGTA CCGTCCGAAG 3180

CTCCTGGAGG AGGACGATGA CCCTGATGAC TCGGAGGTGA TTTGCAGCTT CCTGGTGGCC 3240

CTGATGCAGA AGAACCGGCG GAAGGACCGG AAGCTAGGGG CCAGTCTCTT CACCATTGCC 3300

TTCGCCATCT ACGAGGTGTG TAGTCCTGAT TGGCTCCAGC CCAGGAAACA TACTTTCCCA 3360

GAGAGGACGC TTCCAGGGGC TTCTAGAGGG GCCCTCTGCT TCCTCAATAC CAGTGACCCA 3420

CAGAGCTCCT GGTATCAGGA CCACTTGTGT TTGTAACAAG CAAAAAATAC CAGGGGGGGC 3480

ATTAGAGAGG CAGTGGAGCG GGCCTGGCAG AACAGGTGCC TGGGGGTCAG GCTTCCGCAT 3540

GCGGGCTGCA GTTGCTGGCA TTGCCTTCCG CAGGCTCCTC ATCCTCATTC ACATCTGAAG 3600

CATCTTCCTT TCTGTTTCTT CTCAAGGTTC CCAAAGAGGT ATAGCAGCAG CAGCGGCCAG 3660

CAGTTGTGTG CAGCACTACC CAGGGGGGCC CGAGTCTGTC TGTGGCTCGT CGAGAAGCTT 3720

CCTGGTGGGG TTTGTGGGCA GGACTTGTGA TAGGAGAGGG CCTTGCCTGT TGTTATTTCC 3780

CACTTGCAGA GCAGGTTGCC TCAGGGCATT GCATGACCCA TGACTACCAC CCCCAGGATG 3840

TGCACTTTCT CCCTCGCACC AGACACTGCA CGTCACACAC ATGCCTTTGC ACACTCACCC 3900

TCCTCCACGC TTACAGCCAC ACACACAGTC ACACAGACGC GTTCTGAGGG TGGCTGCCCG 3960

CTTGGGATGG AGGAATCACT TCCCTCAGAA CCCAGCCAAG TCCTCTAGGC CTCCTTGGGG 4020

GTCCTTCCAG CCTGAGGGGC TTCGGAGCTG AGGACAGCTG TTCTGGTAAG TGTCCCTGAG 4080

TGTGGGGATG ACACATTTCC ATTCACTCTG AATCACAACA GAAAAGGGAA GAGGAATTGA 4140

GGTAGGGAGC CTATTTAACC CTTGGGAGTC GGGAAGTAGG GAGGTTGAAA CTGTGACATG 4200

GGTGACCAGG GAGTTGGGAA GGGACCCTTG GAGGTGGCTG TGGCAGGACA GGACGTTCCT 4260

CCCGAGGGGC TCATGTGCCC TGGGCTCTCC CCATCTCTCA GATGCACGGG AACAAGCAGC 4320

ACCTGCAGAA GGACTTCTTC CTGTACAACG CCTCCAAGGC CAGGAGCAAA ACCTACATCA 4380

ACATGCGGGA GGTGTCCCAG CGCTTCCGCC TGCCTCCCAG CGAGTACGTC ATCGTGCCCT 4440

FIG. 8D/3

CCACCTACGA GCCCCACCAG GAGGGGGAAT TCATCCTCCG GGTCTTCTCT GAAAAGAGGA 4500

ACCTCTCTGA GTGAGTGCTG GCCCAGCTTT CCCACGTGTT TCTAAAAGCT CACATGGCCC 4560

ACTCCAGAGG TTGAAGGCAT GAGGCAGCTA GACACGTCTC CTCCAGGGTC CTTCTGCTGC 4620

TCCTGAGCCA CTGGCCACAT TACCCCCATT CATTCATTCA TCCATTCTGT GATATTTATT 4680

GAGCACCTAC TATGTTCCAG GCACTGTCCT AGGCACTAAG GATAGAGTAG TGAAGTAAAC 4740

AGAAAGAAAT CCCTGCCTTC ATGGAGCTTA ATATTCTAAC ATGAGACAAT AATGGATAGG 4800

AAAAACATAT GTAGCATGTT AGATTTGGAG AGGTGATATG GAGCAAAAAT AAAGTAGGGA 4860

AGAGGGATAG GAGGTGTTGG GGATGCTTGA AATTTTAGGT TAGCATGGCC AGGAAAGCCA 4920

CATCCTGTCC CTGGCCACCA CAGATGAGCT CATAGCCCCT GCCACTCTGA TCTCTGTCCT 4980

TGGAAGATGC ACCAGGTCCA TGGGTAGGTG GCTGGGTCAT GCCTTTGGGG GGCTCTGAGC 5040

AATACTAACA AGAACCTGCG TGCCTGGGCT TGGCTGTCGG GGATGGTGCT GACATGGGGC 5100

TGGTTCCTGG GGTTGGGGTG TTCCAGGGGT TCTCTAGAGG CTGGTTCTGG CTTGGCTGCC 5160

AGGAAGCCGT GCACCAGAGC AAACCGTCCA CGGGCCTCCT GCTTGCTTCT GGTGACACTG 5220

AGACCCCACA TGTCTGTATT CCTCACAGGG AAGTTGAAAA TACCATCTCC GTGGATCGGC 5280

CAGTGGTGAG TGGTTTAGAT CTTCTGTGCG AAAAGTCCAG AGGGTCCCCT TCCCTGACCA 5340

TGCAGGGGAC AGATGGTGCA GGGGAGAATG GGCACTGGCA GAGGGAATGG GAGTCTGGGC 5400

TGTGCTGAGC AGTCCCTCCT TGGCACTGCA AATCCTACTT TGGCATGGCC AGAAGTAATC 5460

GGCCTTAAGC ACCGGGGGCC ATTGAGGCAG TTCAGGGGCT GGGAAATATG GAAGAGGGTC 5520

CTGGAAAGGA GAAGCAATTT GAACAATCGG AGGGAACAAG GCCACAGGAA GGGATGACAA 5580

GAGCCGCAGC GAACACTGGA TTCTGAGACT GGATAACATT GGATTTCACA CATAGAGAAA 5640

AGAAAGTAAG CTGGTGCCGG ACCTGGTGTT GACACTTGGA TCCTCCACTT ACCAGCGGGG 5700

TGACCTGGAC AATTTCTGTA ATCCCTCTCA CTCAGTTTCC TACTCAGTAA AACGGGGATG 5760

ATAATGTGCC TTGCAAGGCT TTTGTGAGGC TTCATCAATG AGGTGATGTA TGTGAAGTGT 5820

CTGGCACAGC ATGGGCACTC AAACAGAGGT GCTTTTTCAC ACTTTACACC TTACAAGGTA 5880

CTTTTCACAT GTGTCATCGC GATACTTGCA AGGTTGCTGA GAGGTAGATG GGGTTATAAT 5940

CCCTGGTGTT CAAGAAAGGA AGCAGAGGCT CAATGGGGTT GAATGACTTC TCTGAGTTCA 6000

CAGAGCTCAG TAAGTGGCAG GGTTTGGAAC TCACATTCAG ACTCTCTGAC TCCAGACTTA 6060

FIG. 8D/4

GGTTTTTCCG CACCTCCACG CTGAGGCCAG CCCCAGGCAG TGAGAAGCCC AAAGTCCGAA 6120

GCACAGAGTG CTGTGTGTTG GGCTCTGTGT GTTGAGGAGT CTTGTGACTG CCTTGGGGCT 6180

TTGGGCTGTA GTCAGCTGAC AGTCCTTTGT GCTCTGTGGG GATGACGTAG GCCAATGGGA 6240

GGACAAATGC CCCTCTGAAC TGTCTTCTGG GCAGTGACAG TCATGGTCAT AATCCTGACC 6300

CTGAGCCAGT GCCAGGTCTC CAAGTGCCTT CTGAATGACC ACAGGCGATT GGTTTTAGTG 6360

GTAGGTGCGT GGGGATCTGT TCTGGTCATC TGGATGCTGG TCATCGGGTG CAGTATTGAT 6420

CAGGACCTGC AAACCCAAAA GCTTATGGGA GCTGGCACGT CACGTGAGTA GAGCAGGCAG 6480

GTGCAGGGTT TTTGATGTCC CTGCACTGAC ACAGTTGTCT GCAGTTCTCC AATTTGACAT 6540

TTGGGCTCCA GTGTCGAGGG TCAAACAAGG AATTTTGGGG CGTGGGCCAA ATCTGGGAAG 6600

ACACAGGGAG CAGGGCCCTT TGGCTCAAGC TGATAGTTGC CGCAGGGATT ACCAGGCCCA 6660

GGGCAGCCTG CCACAAGCTG GGGCTTTTAC CAAAGAAAAT CTCCCTATGT TAAATGCTTG 6720

CTCAAAAATT TTTAAAAAAT ATTCTGTAAG TCAAAATCCA TTGTTAGGTC AGTTTGAGAG 6780

AGCCATGTTT TTGGTGTTTT AGTAACCAAT TTCATTTTTT TATTATTTAT TTATTTGTTT 6840

ATTTTTGAGA CGGAGTTTCA CTCTTGTCAC CCAGGCTGGA GTGCAATGGC ATGATCTCAG 6900

CTCACTGCAA CCTCCGCCTC CCGGGTTCAA GCAATTCTCC TGCCTCAGCC TCCTGAGTAG 6960

CTGAGATTAC AGGTGCCCAC CATCACGCCT GGATAATTTT TGTATTTTTT AGTCGAGATG 7020

GGGTTTCACC ATGTTGGCCA GGATAGTCCT GAACTACTGA CCTCAGATAA TCCGCCCACC 7080

TCAGCCTCCC AAAGTGCTGG GATTACAGGC ATGAGCCAGC ACGCCCGGCC ACCAATTTCA 7140

TTTTTTAAAA AAGGAAGAAA GAAAACCTTA GCCAGAAGAT CTTTTTCCTT GCCATATGCA 7200

GTAAGAGTAG ATTATAAAAA CAAAGTCAGA GCAGTCACTG GTGTCTGGGC ATGGAGGAGA 7260

AAGAAGAATT CTCTTCTCCC TTCACCCTCC ATGCCCCTTT TTGGCTCCAT GTGATTCAGA 7320

TTTCTGGACC CTGGAGCCCC ACCCCAAGCT AAAGACCAGG ATACAGGGAA GCCACAACCA 7380

CTGGCGGTTC TGAGAACTTA CTTTTCACTT ATTCTGCATT TACTGTTTCC TTTTCTTATG 7440

CAGAAAAAGA AAAAAACCAA GGTAGGTGTG TGGGTAGAGA GCATGAAGTG TGTGTACTCA 7500

TGCATATGTA TGTGCATGCA TGTGAAGTGT GCATGTGTGA GCTCATATGC ATCCATGCAC 7560

CAGACTTGCC TCTTCCTCCC CCTCCTTCCT GAGCTTCTGC TGGGGCCGAG CGTGCAGTAA 7620

TGACAACTAC GATTTGCTGG GGGAAGGCTA CGTGCCAAGC ACTCTTTTAG GTGCTTTCCA 7680

FIG. 8D/5

TGATTAATTC CTTCCTCACA ACAGCCCTAT GAGATTAGTA CTATAACTAT CCCCATTTTC 7740

AGAGGGAGAA AAGGTACAGA CTTGACTAAC TTGCCCAAGG CCACACAGCC AGAGAGGGGC 7800

AGAGCCAGTA CTTAGAGCCA GGCAGTCTGG GTCCAGAGTC CGTGTCCTGA ACCACAAGAG 7860

GCCATCATAC GCCATCAGAT TTGGTGCTAG CATTTCTGGT GGTGCCTGGT GGTGATGGAT 7920

CCATCACAGG GGTCCTCCAG GTACTGGTGC TGGCCCAGAC CAGAGCTGAC ACTCCTCAGG 7980

CACTACCACA TTCCAGGCAC TGTGCTTGGG GTCAGTCCCT CTCTTTTTTT TCCCCCCCAA 8040

TTATAACAGT ATCTACAAAG TAGGTGCTGT TATTTTTCCC CTTTCACAGG TGAGATAGAC 8100

TCAAAGAAGT GAACTTGCCC AAGGAACAGA ACTAATGAGT GGGGAAAATG GAACTGGAAA 8160

CCATGTCTGT TTACTCCAAA ACCTGTGTTT CTTGCCCTCT TTCTCTGATG CCAGCCCCCT 8220

ACACTTCAAG GCCTGTGTTG TCCAGACCCA CACTCGGGCC TGCCAGTGTG TGCCTGGCAG 8280

GGATGCTCCA TGGCCACACC ATATCCATCC TACACATCCC CCCTCAGACT GTGACCTCCA 8340

TTTGCTCTGG GATCCCCACA AGCTTCAGCT GCTTGAGCAA GACACTGCTT AGAAGGCAGA 8400

GCAAGCCAAG GCCTCTGGGG CCTGCTGGGA GCCAAAGCTG GGGAGCCGTT TCCACGGGTC 8460

TATCTGCTTG AGCTGTCCTA GATGAGCAGC ATGGAAGGGC AGTGGTGCAT GAGTCCAGGC 8520

GGGCTGCTTT TCTGCTCCGA GAGGCTCTGC CTGCCCAGTT GTTCTCTGCA TTGCAGCCTC 8580

AATCCCCACA GCCTTGCCTT CCCCCGGCTT TCCCTACAGG TGCACCGCAT CCACAGTGTT 8640

GGCACCATGC AGCAGCCGCT CTCCGTCCTT TTCATATCCT TGTCACTTGC ACGAGCATGT 8700

CTTGAAAATA TCCCTTGTTT GTGTAGCATC TTAAATGTTT TTGCAGTATG ATTTTGCATT 8760

CAGTATCTCA TTTGATCCCC ACAAGAGCCC TATGAGGAGG GAAAGCAGAT TTTACCATTA 8820

AAGGATGAGT AAACTGAGGC CAGAGAGGAT ATTTTTGGTT TTTTTTGAGA CAGTCTCACT 8880

CTGTCACCCA GCCTGGAGTG CAGTGGCTTG ATCTTGGCTC ACTGCAAGCT CCACCTCCCA 8940

TGTTCACACC ATTTTCCTGC CTCAGCCTCC CAAGTAGCTG GGACTACAGG CACCCACCAC 9000

CACACCCAGC TAATTTTTTT GTATCTTTAG TAGAGATGGG GTTTCACCCA GTTAGCCAGG 9060

ATGGTCTTGA TCTCCTGACC TTGTGATCTG CCTGCTTCGG CCTCCTAAAG TGCTGGGATT 9120

ACAGGCGTGA ACCCCCCTGC CCGGCCAGAG AGGATATTTC TTAATGAGGG GCAGGGCTGG 9180

GATTCCAGCC CAGTGTTCTG ATGGCTCACC CACTGACCAT TCCACTAATC CGTGTCCTTT 9240

TTCAATCTAA ACTTTCAGGG TTGTAGAGGT TCCTTTGAGG TGCCTCAGTA CTTCCATGGT 9300

FIG. 8D/6

GATGTGGGGT CTGAGCGCCA AGAGCTCTGT TCTCATTAAT CAGAGAAGCT TGTGTTTTTA 9360

AAAACACCAT GTTTACTGCA GGAAATTTAA TTGGACAGTG TTTCCATCTG GAAAAAAAAA 9420

AGTCTACAAA ATACTTGACA ATCACTGCAC TAGATCATGC TGCTTTTAGC ATTCTTAGCA 9480

TTTCACGTGC TGAGCTCTCA ATACTCTACC ATGAGGAGGG ATGGAGTGGG TATGAAAAGA 9540

TAAAGAACTG AAGTCACACG GCTTGTCAGT GGCAGAGATA GAGCTTGAAC CGAGGTTGAA 9600

GAGCTCCCGC CTATTCCTTT CCTCTTCTCA CTGGATAAAG CTGCTCCAAG AGAGGTGCTG 9660

CCTCAGTGTG CCTGTTCAGA CTGTAATCCT CCCTTCCTTC CTGCCTCCTC CCTCCTCTCT 9720

CCAGCCCATC ATCTTCGTTT CGGACAGAGC AAACAGCAAC AAGGAGCTGG GTGTGGACCA 9780

GGAGTCAGAG GAGGGCAAAG GCAAAACAAG CCCTGATAAG CAAAAGCAGT CCCCACAGGT 9840

GTCTGGGCAT GTGGCATGGG TGGGGTGGCC AGCACGCTAC AGGGGCTTCC TATGCGCTTG 9900

GGATACACAG GGGCTGGAGG CTTCCCAGGA GTTTGTCTTG AACATCTGGA GGTTTGAATT 9960

TGTCCCACTG ACCTTTTCTT TCAGCAAGTT CCCCTGAAAT TTGGGCTGCT GCTTGGGTGA 10020

ATATCCCAGG ATGGGGGTTC CATTCTAGGA GTGGACTGGC AGGCTGAGCC TCCCATGGAG 10080

CTGATCCAGC CAGGATACAG AGAAGGGGAG GCAAAGGCTG AGACAGAACC AGCTTGAGAG 10140

CGGAGGCGCA ACTCTTGTCT CCTGGTGGCC TTGAGCATTT CACAATAGGG GGATAAAGGA 10200

TAGGAGCAGA AAAGTGGGGC TGACTTCAGA AATGGGGTCC TCTAGAGCTC ACGGGAGGGT 10260

GTTAGATTGG AGTGGGAGCT TAGTGGAGGT GAGCCTTAGA GGCAAAAGTC TCCAGACCAA 10320

TCCAGGCCCC CTCTTCTATC CGGGGGCCCC TCTTCTATCC AGGGCCCCTC TTCTGTCTGG 10380

GAGCCCCTCT TCTATCTGGG GCCTCATGCA GTGGGGCCTA GGGGAGGTTC TCTGAGGACT 10440

TGGCCTTGAT GACAGGGTGG CTGGAGGAAT CAGAACGGTC AGACCTTCTT TGACCTGCGG 10500

GCACCTTTAG TTGGAATGCT CAGGCCTGGG ATGGTGGAGG GGGCTCTTGC AGGTGGGGAC 10560

TGGGGTGGCG GGGAGGAGGC TGTATGGCCG CCATATCTCC TTTGGCTGGG GGCGTCAGGG 10620

CTGGAGAGGT GTGAAGAGTC CCTGAGGCCT CGATGCATCT CACTCCAGCT CACCAGGTCT 10680

GCATTTGCCC GTCCCCAGCT CCTGCTGCCA CCCCCGGCCG TTTTAGGCAC TTGGCTCCCT 10740

TGGCCCAGAG GAGCTTGCCT CACAGGCCTG TGCACCTCTG ACCCCTGTGA ACCAGTTTTC 10800

CTTTGTGCCT CCACAGCCAC AGCCTGGCAA CTCTGATCAG GAAAGTGAGG AACAGCAACA 10860

ATTCCGGAAC ATTTTCAAGC AGATAGCAGG AGATGTGAGT ACCTCCAAGC CCAGGACGCC 10920

FIG. 8D/7

CACAGGTGCT TCCTTCTCTC CTGGATTAAC TGCTCAGATT ACCAATTATT TCATTATTGT 10980

TTGGTAGAGG TCACTTTGGA CTTCGGTGGA GCCAGGGGAT GTGTGCGTAG CACACAAATC 11040

CACAAGCCCT TGAGTTTTGG ACTGCCACGT CTGCTGGGGG GCTCAGAGGC CTTTTTGCTC 11100

TGAGCTGCCC ACGGTGGTCC TGATAGCTGA GGTGCAGTAT CTGGCCCCCT GTCTTCCTCA 11160

GAAAAGCCCC AGCTTCCCAT GACATAATAG CACCGACAGG GATTTTACAA ACACAGCCAG 11220

GTGGAATTTG TTTTGCAAAG TGTCCGCGCC AGGAGCTGCT GTACTCCTGA ACCATGACCC 11280

TCCTCTCCCT TCCTCCTCAG GACATGGAGA TCTGTGCAGA TGAGCTCAAG AAGGTCCTTA 11340

ACACAGTCGT GAACAAACGT GAGTTGCTCA AACCAAATGG GGGTGGGGTG GGTGGGGAGT 11400

CCCGTTGTCT CAAAGCAGCT CCTCACTCTT CTCCATCCCC CCAGACAAGG ACCTGAAGAC 11460

ACACGGGTTC ACACTGGAGT CCTGCCGTAG CATGATTGCG CTCATGGATG TATCCTTCCT 11520

GCCGCCCCTT CCCGACCCTC TGTCATCAGC CCACGGGGGC CAAGGCAACA TACAGGGTGC 11580

CCAGTCAGGC AAAGGGCCCT AATTTGTGCC CAGGGAAACT TAAGGAGACC CTGATTCAGA 11640

ACATCTTGGA TACTCGTCTG AAAGGGGTTG TTAGAGGCGG AAGGGGAGGA TGTTGGGTTG 11700

TAACTGCCCT AACCCCTGTG CTTCTCTCAG GCCTGGGATC CTGCCCAAGC AAAAGTGGTC 11760

CTTAGGAGAG CGGCTCCTGG GTTACAGAGT AGGCGCAATC TCTGACTGGT GGTGGAGTGG 11820

AGGGGAGGGT TAAATAGTAC AACAGGGCAG TGGGTAGGAC AGCCCGGAGT CTCCTAGACC 11880

CTCCCTCCAA ATCCAGGGGG ATTTTGCTGT GTGCTGTGTA GCCCTGACCT CCCTCCTCCA 11940

GACAGATGGC TCTGGAAAGC TCAACCTGCA GGAGTTCCAC CACCTCTGGA ACAAGATTAA 12000

GGCCTGGCAG GTGGGAAGAG AAAATGAAGC GTGGGAGTCA AGAATGGGGT TGATTTGGAG 12060

ATTCAGTGTG TGACCTCCAT CCTCAAATTT TCTATTGCCA GAAAATTTTC AAACACTATG 12120

ACACAGACCA GTCCGGCACC ATCAACAGCT ACGAGATGCG AAATGCAGTC AACGACGCAG 12180

GTGCTGAGAA GGAAGGGGTG TCAGGGATGT GGACCCGAGA CGGTGGGAGC AGGAATGGGA 12240

GGGGACTAGC TACTAGGGCC CCACTAGAGA AGGAGAGGGA AAGGGCTTCT CACTTTCCCT 12300

TCCCAGGTCA CAGAGTGTCC GAGAGGCAGG GAAAATAGAA GACAGGCCCA AGGCCTCCAG 12360

CTCCACGTCC ACCTCTAACA TGGTCCCCTC CACAGGATTC CACCTCAACA ACCAGCTCTA 12420

TGACATCATT ACCATGCGGT ACGCAGACAA ACACATGAAC ATCGACTTTG ACAGTTTCAT 12480

CTGCTGCTTC GTTAGGCTGG AGGGCATGTT CAGTAAGTGG GAGAGGGGGG CTGCCCTCTG 12540

FIG. 8D/8

CTCTCTTGCA GGGGCAGTTG TGGCAACAGG CATCTCACCT GATAATCTCC AGTCTGCTCC 12600

ATCCAGGCTG AACAAGGGCC AATGACCTCT TTAGGCCCAG AATGGGATGG CAAAGGGAGG 12660

GTTACTGGTG ATTCTCTGCC TGCACATCTT TGTGCTGATG AGGGACAGCA CTGGGCACAC 12720

GGTCCTCTGA GGGGAAGTTA CAGTAGTAGA GGCGGAGTGC GCCTGTAACT GGCCTCTGGC 12780

CTGTGCATTC TTTCACAGGA GCTTCTCATG CATTTGACAA GGATGGAGAT GGTATCATCA 12840

AGCTCAACGT TCTGGAGGTA AAGCATAGGC ACAGCACATT CCCCCTACAC ATTAAAACTC 12900

AAGGTGGAGG GGTCAACGGG GCGGACTGGA CCCAGGGTGT GCTCCTCATT TCCACACAGT 12960

GGTGGAGGGA AGGGATAGGA ACAGAACATG GAGGGAGGCT CAGCAGGCTC CCAGGACACA 13020

TGCACTTGAG GCCCAAAAGG ACCTCTGCTC CCCCAGTCAC TTGATGCGGG AAAACATGCA 13080

CCTTCTTAGG GAAGATCTAG GAGAAAGGAA ACAGTAAGCC ACTGCTTCTT GGAAAATCTT 13140

CTGGGGGTCT GACCTGCTGG GACTGTTCCC TTTCCTCTTG CCCCGTAAGA TTCCTAGGGC 13200

GGGGGGGGGG GGGGGTCACT CTTTTCTGAT CTACATTCTG ATCTTGGGAC TTCTTTCAGT 13260

GGCTGCAGCT CACCATGTAT GCCTGAACCA GGCTGGCCTC ATCCAAAGCC ATGCAGGATC 13320

ACTCAGGATT TCAGTTTCAC CCTCTATTTC CAAAGCCATT TACCTCAAAG GACCCAGCAG 13380

CTACACCCCT ACAGGCTTCC AGGCACCTCA TCAGTCATGT TCCTCCTCCA TTTTACCCCC 13440

TACCCATCCT TGATCGGTCA TGCCTAGCCT GACCCTTTAG TAAAGCAATG AGGTAGGAAG 13500

AACAAACCCT TGTCCCTTTG CCATGTGGAG GAAAGTGCCT GCCTCTGGTC CGAGCCGCCT 13560

CGGTTCTGAA GCGAGTGCTC CTGCTTACCT TGCTCTAGGC TGTCTGCAGA AGCACCTGCC 13620

GGTGGCACTC AGCACCTCCT TGTGCTAGAG CCCTCCATCA CCTTCACGCT GTCCCACCAT 13680

GGGCCAGGAA CCAAACCAGC ACTGGGTTCT ACTGCTGTGG GGTAAACTAA CTCAGTGGAA 13740

TAGGGCTGGT TACTTTGGGC TGTCCAACTC ATAAGTTTGG CTGCATTTTG AAAAAAGCTG 13800

ATCTAAATAA AGGCATGTGT ATGGCTGGTC CCCTTGTGTT TTGTTGTCTC ACATTTAGAT 13860

ATCAGCCATG CATGACTGAA TGGCTTCCAA TCATATACTC ACCTATCACC TACAAGAGAA 13920

CAATGAAAAA CACACACAAA AACAAAATCT TGAATTTTGT AATCATGCCT ATTGCTATTT 13980

CTTGAGCATA AGAATGGCTC AGATACTTTC CAAGACATAA AAGGAAGGCA GAGGAATAGT 14040

TGTTGCTGTA AAAGACATCA AGAATAAATG GGGTCATGTA CAACGGGAGG GGCCGGTTAC 14100

CTGAATAATG GAGTGGAGAT TGAGCTATCC TAGCTCCTCT GCTCACTAAC TGACCTGTCG 14160

FIG. 8D/9

CATGACCGTG GACAAAACCC TGAACGCAGC TGTTTGTTTG CTAAACTTCT CTGGACCATG 14220

GCCTGCGGCA TATCTATAGG CATCCTGTGT TTTCCACCCA GTTTCCTTCT TCCTCGCTAA 14280

GCCAACGTGG AAAGGGCTGG CCGTGAATAT GCAGACAAGG TAACGAAAGT AAACCGTCAA 14340

TTAGTAAAAG TACTTCATTT TCCTCTTGTA TTTGCTTCAT TCTTGCTTCA CAAAGTTACG 14400

AAGTCCACAG CTTTATACCA AAATGTAAGA AGGCTATTTG CTTATAAACA TTTTGAGTCA 14460

GGTGTCATCT GATTTCATTC TTCTAATCCA TATTCAATAT TAAAAAATCA GAAACCAAGG 14520

GTGCTGGAGC AGCTCTAGGG CATATATTTC TCTTAAATAG GAGAAAGATT TTCAACAGCT 14580

TTTCCTCCTT GACCCCCTCC TTTCCCAATT TATTTGGGTC ACTACCTTGA ATTTAGAGTG 14640

AATCTGGGAA ATGTAGTCAC CAGG 14664

FIG. 8D/10

960-29

Nixon & Vanderhye P. C. (12/95)

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

LGMD GENE CODING FOR A CALCIUM DEPENDENT PROTEASE

the specification of which (**check** applicable **box(s)**):

- [ ] is attached hereto
- [ ] was filed on _____ as U.S. Application Serial No. **(To Be Assigned)** **(Atty Dkt. No. 960-29)**.
- [X] was filed as PCT international application No. **PCT/EP95/04575** on **21 November 1995**

and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Prior Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
94402668.1	Europe	22 November 1994

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	Day/Month/Year Filed

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s): Application Serial No.	Day/Month/Year Filed	Status: patented, pending, abandoned
PCT/EP95/04575	21 November 1995	Pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Paul J. Henon, 33626; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr., 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; William J. Griffin, 31260; Robert A. Molan, 29834.

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